The Journal of Laboratory and Clinical Medicine

VOL. 19 ST. LOUIS, MO., APRIL, 1934 No. 7

CLINICAL AND EXPERIMENTAL

EFFECTS OF LOW TEMPERATURE RETARDATION IN THE CULTURE OF STERILE MAGGOTS FOR SURGICAL USE*

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A LARGE number of laboratories, chiefly connected with hospitals, are now engaged in the culture of blowfly maggots for use in the Baer method[1] of treatment of infected wounds. In the cultural process it is necessary to hold back the rapid development of certain immature stages,[2] and it appears to be customary to do this by means of low temperature. Owing to the widespread use of this method of retardation and to observations made in the Bureau of Entomology which indicated that this procedure has certain limitations, an investigation has been made of the general effects of such retardation upon the normal functions and mortality of all stages held back in this way. A mechanical refrigerator running at from 40° to 43° F. was used. This range was found to be low enough to retard development, and is probably accessible to most laboratories. *Lucilia sericata,* the species commonly reared for the maggot treatment, was used in these experiments.

THE EGG

It is the usual practice to remove eggs from the fly cages in the afternoon and place them at once in cold storage for disinfection the next morning. It frequenty happens also that a surplus of eggs is laid and they are stored until

*From the Division of Insects Affecting Man and Animals, United States Bureau of Entomology.

Received for publication, July 3, 1933.

needed. The length of time eggs may be retarded without injury, under various methods of storage, was tested in this series.

Source of Eggs.—All eggs used were produced under uniform conditions of temperature, moisture and food. Also, since the age of the female affects the viability of her eggs, the females were used only during the first three weeks of oviposition. Eggs were taken within two hours of deposition.

Methods of Storing.—(1) Eggs were left upon the meat and placed in covered Petri dishes. (2) Eggs were removed from the meat and placed in vials of water. (3) Eggs were removed from the meat and placed upon moistened cloth over a wet cotton pad in a covered Petri dish.

Since the eggs are usually laid in masses and are difficult to count, the weight method[3] of determining number was used. Eggs were removed from storage at intervals up to 3 days and given a viability test.[3]

Effect of Age of Egg upon Survival.—It soon became evident that the degree of development of the embryo at the time of storage of the egg was important (Table I). When eggs freshly laid and up to two hours of age were

TABLE I

EFFECT OF AGE OF EGG UPON SURVIVAL IN COLD STORAGE

AGE WHEN STORED (HOURS)	NUMBER OF EGGS USED	NUMBER OF TESTS MADE	SURVIVAL (PER CENT)
0 – 2	3,623	6	27.6
1 – 3	2,134	3	73.1
2 – 4	3,138	6	82.1
3 – 5	2,009	3	82.5
4 – 6	3,910	5	74.8
(Normal* hatch, 85 per cent)			

*The term "normal" used in each table refers to results when specimens are allowed to develop as checks in the ordinary fly cabinets without being stored.

placed in storage for twenty hours, only 32.5 per cent of the normal hatch occurred, whereas eggs two to four hours old when stored produced 97 per cent of the normal hatch. Therefore, only eggs which had been laid two to four hours were used. They were obtained by removing the meat from the fly cages after two hours and keeping it in the warm incubator for the additional two hours before storage.

Results.—Cold storage has a destructive effect upon the egg, as shown in Table II. Even the twenty-hour exposure, which is only slightly greater

TABLE II

LENGTH OF TIME EGGS CAN SURVIVE COLD STORAGE

TIME IN STORAGE (HOURS)	NUMBER OF TESTS MADE	NUMBER OF EGGS USED	SURVIVAL (PER CENT)
20	9	2,603	67.4
28	9	2,742	66.5
36	9	2,588	48.3
45	9	2,366	40.1
58	9	2,423	18.2
72	9	1,489	1.8
(Normal hatch, 77 per cent)			

than the customary overnight storage, reduced the normal hatch by 12.5 per cent. The mortality was increased rapidly with length of exposure, and after three days' storage almost the entire number was destroyed. No difference in effect was observed among the three methods of storage. Mortality was greatly increased when eggs were removed directly from the fly cages to the refrigerator.

THE SURGICAL MAGGOT

Owing to the irregularity in the demand for sterile maggots a surplus frequently occurs. The expense of production would be reduced if any surplus could be stored safely until needed. Experiments were therefore conducted to determine the mortality under cold storage. The name "surgical maggots" is applied here to sterile maggots ready for use in wounds and about 4 to 6 mm. in size.

The maggots used in these tests were produced under the same sterile conditions as for use in wounds,[2] but in smaller lots of about 200 each. They were stored with some food in glass rearing bottles[3] from one to six days.

Survival Tests.—As a check on their tolerance of cold storage, it was required that the maggots should be able not only to move when restored to the warmth of the laboratory but to resume feeding and growth. It is obvious that maggots which cannot feed are of no use clinically. Accordingly all maggots living when removed were placed upon raw beef to test feeding capacity.

Results.—A high rate of mortality occurs in cold storage of surgical maggots (Table III). The last column shows that even when stored only one

TABLE III

LENGTH OF TIME SURGICAL MAGGOTS CAN SURVIVE COLD STORAGE

TIME IN STORAGE (DAYS)	NUMBER OF EXPERIMENTS MADE	NUMBER OF MAGGOTS USED	NUMBER WHICH SURVIVED STORAGE	NUMBER ABLE TO RESUME FEEDING	SURVIVAL (PER CENT)
1	7	3,006	2,655	1,104	37.9
2	6	2,764	1,650	922	33.3
3	6	2,823	1,112	604	22.2
4	5	2,183	781	397	18.2
5	4	985	391	119	12.2
6	2	448	55	7	1.5
	(Normal survival, 81 per cent)				

day less than 40 per cent were able to resume feeding. The mortality, increasing rapidly, amounted to almost 100 per cent in six days. Maggots reared under the same conditions but not stored had a normal mortality of only 19 per cent. Death of surgical maggots under the usual method of cold storage is therefore very considerable.

The fact that cold storage is very injurious to surgical maggots is evident not only in the number of maggots which were killed outright while in storage, but also in the inability of about one-half of the survivors to feed and continue their development. These latter losses easily escape detection unless closely observed. Cold storage of surgical maggots should therefore be avoided.

A Method of Avoiding Cold Storage of Surgical Maggots.—The only time that storage of maggots is essential in the cultural technic as ordinarily practiced is during the sterility tests,[2] when the growth of the maggots is held back in the refrigerator for forty-eight hours. Such retardation of growth by low temperature, however, is no longer necessary as a food has been devised by one of us (S. W. S.) which prevents the maggots from growing too large during the sterility tests. This food permits only a slow rate of growth throughout the cultural period, but does not interfere with normal feeding of the maggots when implanted in the wound. It consists of 1 part of evaporated milk to 5 or 7 parts of water, to which is added 1.5 per cent plain agar. This mixture is cooked in a double boiler for about twenty-five minutes and approximately 15 c.c. is used in each food bottle.

PREPUPA

This stage is a convenient one for storing, as the larvae have already migrated from their food and are easily collected and handled.

Method of Storing.—Prepupae were taken from the sand about thirty-six hours after migration and while they still resembled larvae. The containers used were the common oiled paper or fiber cans, half-pint size, which are readily obtainable. They are waterproof and have a fairly tight-fitting lid. About one inch of dry sand was placed in each container along with the specimens. If a much larger quantity of sand is used, it should previously be chilled to storage temperature, otherwise the prepupae tend to develop to the pupal stage before the sand is cooled. It is important to prevent such development, as pupae are much less tolerant of storage than prepupae, as will be shown in the next section.

Loss of Weight During Storage.—Preliminary tests had shown that when no special precautions were taken to provide air-tightness in the containers, a noticeable loss of weight of prepupae occurred in storage. During the present experiments an investigation was made of the cause of shrinkage and methods of prevention. Specimens were stored in the containers under three different degrees of air-tightness. (1) Container was sealed with adhesive tape around the edge of the lid. (2) Container was not sealed and the lid fitted in the ordinary manner. (3) Container was the same as No. 2 but had numerous pinholes in the lid.

Each time specimens were removed from storage for survival tests, an equal number (twenty-four) were taken out and desiccated to constant weight at 100° C. for determination of weight losses.

Survival Tests.—Removals from storage were made at the intervals shown in Table V. The mere ability of prepupae to revive and move in the warm air of the laboratory was not considered sufficient indication of survival. Development into adults and oviposition of viable eggs by the females were considered necessary parts of the test. Accordingly, each lot when removed from storage was placed in a rearing cage, and the necessary care was given up to oviposition. When eggs were laid a viability test[3] was made in each case.

Results.—The percentage of water and solid losses under the three methods

of storage are given in Table IV. Loss of water began at the outset of storage and continued throughout the period. Specimens stored in the sealed containers lost the least in both water and solids.

As to survival from storage, Table V shows that in two to four weeks no considerable mortality occurred, as 15 per cent is the normal death rate without storage. Holding beyond four weeks, however, caused a rather high death rate, and after nine weeks the mortality was 60 to 70 per cent.

The lowest mortality always occurred in the sealed containers. There is therefore a correlation between death rate and loss of weight in storage. Apparently when the water content is decreased below 33 per cent the mortality becomes especially high.

TABLE IV

EFFECT OF METHOD OF STORAGE UPON LOSS OF WEIGHT OF PREPUPAE

TIME IN STORAGE (DAYS)	PERFORATED CAP		ORDINARY CAP		SEALED CAP	
	WATER (PER CENT LOSS)	SOLID (PER CENT LOSS)	WATER (PER CENT LOSS)	SOLID (PER CENT LOSS)	WATER (PER CENT LOSS)	SOLID (PER CENT LOSS)
7	10.4	0.2	12.1	0.2	4.2	0
14	14.7	0.3	15.6	0.3	9.8	0
21	19.9	0.4	17.6	0.3	15.3	0
35	24.8	0.5	23.9	0.4	22.1	0.3
49	32.3	0.7	34.0	0.8	31.0	0.7
62	38.2	0.9	38.5	1.0	36.6	0.8
76	44.4	1.9	44.4	1.9	41.1	1.1
91	46.4	2.3	48.3	2.2	42.1	1.5
105	48.3	2.3	50.0	2.3	43.4	1.8

Another unfavorable aspect of cold storage of prepupae, which is not readily apparent, is the reduced egg-laying capacity of surviving females. In 9 colonies of flies which had been stored from three to four weeks as prepupae, it was found that the length of life of the flies was only one-half that of the preceding generations which had not been stored, and that the number of eggs laid was reduced to one-third of the normal.

TABLE V

EFFECT OF COLD STORAGE UPON PREPUPAE

TIME IN STORAGE (DAYS)	PER CENT SURVIVAL		
	PERFORATED CAP	ORDINARY CAP	SEALED CAP
16	83.5	67.8	83.5
33	60.5	54.2	66.6
49	45.8	45.8	62.5
62	29.1	29.1	37.5
76	18.3	24.1	37.5
91	18.3	19.1	20.8
105	12.5	8.3	12.5

(Normal survival, 85 per cent)

THE PUPA

Pupae, being especially easy to handle and store, and requiring no food or attention, constitute a very convenient stage for storing. Reserve stock is frequently held in this stage.

Method of Storage.—The pupae were used when about thirty-six hours old. They were placed in the same three types of containers as used for prepupae. The same standard tests for survival were also made.

Results.—It soon became evident, however, that pupae could not be satisfactorily held over, as they were unable to endure even a moderate period of storage. After the second week the emergence fell to almost one-third of the normal, and most of the eggs which were laid failed to hatch (Table VI).

TABLE VI

SURVIVAL WHEN STORED AS PUPAE

TIME IN STORAGE (DAYS)	SURVIVAL (PER CENT)
7	66.6
15	37.5
22	15.2
29	11.1
36	8.2
43	2.7
(Normal survival, 86 per cent)	

After three weeks of storage the females which emerged died at an early age and laid no eggs. The type of container appeared to have no effect upon the results obtained with pupae.

SUMMARY AND CONCLUSIONS

In the production of surgical maggots it is customary to retard the rapid development of the immature stages by holding them in reserve under cold storage. An investigation has been made of the tolerance of the egg, surgical maggot, prepupa, and pupa to various periods of retardation.

Eggs may be kept in a closed container in a refrigerator at 40° to 43° F. overnight and up to twenty-eight hours with a mortality only slightly greater than normal, but continued storage is very unfavorable, and in three days results in almost complete destruction.

The age of the egg when stored affects the degree of injury. The lowest mortality occurred when eggs were allowed to remain in the warm incubator an additional two or three hours after removal from the fly cages before being stored.

Surgical maggots cannot satisfactorily be kept in cold storage. After twenty-four hours' retardation only 40 per cent were able to resume feeding, and in six days almost 100 per cent destruction occurred. A retarding food is described which eliminates the need of cold storage during the sterility tests.

Prepupae are best adapted to cold storage, but even in this stage the possibilities are considerably limited. They lose weight and become shrunken during cold storage. This is mostly water loss. Least unfavorable results were obtained with a relatively air-tight container. In thirty-three days the mortality was from 33 to 46 per cent and in seven weeks it had increased to approximately 38 to 55 per cent, depending upon the method of storage.

However, when prepupae were stored from three to four weeks the females which emerged were permanently injured and their egg-laying capacity was much reduced.

Pupae are unable to withstand even a moderate period of retardation. After the second week, mortality rose to about 66 per cent and most of the eggs subsequently laid failed to hatch.

Low temperature retardation of the various stages in the life cycle of the blowfly, although a convenience in cultural technic, causes such a high mortality that its use is considerably limited. It appears to be best adapted to short storage periods or when the convenience of continued storage is sufficient to counterbalance the losses.

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BACTERIUM MELANINOGENICUM*

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SINCE the brief description of *B. melaninogenicum* by Oliver and Wherry,[1] in 1921, there have appeared several studies of this organism. Attention has been focused on the subject because of two established facts, that the organism produces an abundance of a dense black melanin-like pigment, and that it is found in a variety of situations in the human body, both normal and pathologic. Pulmonary abscess is only one of the various pathologic conditions in which the organism is frequently encountered, but it is an example of a number of processes in which the etiology is obscure, and in which the developed affection represents more than likely a complex secondary infection. Since relatively little has been reported on *B. melaninogenicum*, since it normally exists in various parts of the body and thus is present when opportunity to invade exists, since it acts on blood substance, and since studies of pulmonary abscess here and elsewhere frequently revealed this organism in the abscess, the study of the type seemed to offer one of a number of possible avenues of approach.[2, 3, 4, 5] The possibility of primary invasion was open. The potential rôle in infections in which the organism was a secondary

*From the Department of Bacteriology and the Department of Surgery, University of California Medical School, San Francisco.
Received for publication, May 3, 1933.
Aided by The Lillie Spreckels Wegeforth Donation for the study of pulmonary abscess.

invader seemed worth study. The concept of the etiology and development of pulmonary abscess has been considered elsewhere.[6]

The purpose of the present report is to indicate the biologic characteristics of *B. melaninogenicum* determined in the course of the investigation above mentioned. It will be apparent that no evidence has been observed which would lead one to suppose that this organism is a significant etiologic agent in what becomes a mixed infection, nor that it has any significant part in the complex changes which obtain in the developed focus.

MORPHOLOGY

The organism is generally described as a small coccobacillus, gram-negative, nonsporulating, and nonmotile. In smears directly prepared from clinical material it frequently appeared as an encapsulated diplococcus (C. K.). In liquid medium pairs or clumps of the typical small cells appear, along with narrower rod forms and slender, slightly bent filaments, occasionally ten or more times the ordinary length. The organisms are somewhat larger and more definitely rodlike when grown on solid medium, and the morphology is more nearly constant. A considerable number of observations seem to indicate that the smaller rod forms take a bipolar stain or appear vacuolated. The cells are not acid-fast. Although measurements are likely to be misleading, those made on what was considered to be a normal or typical smear indicated a length of from 0.5 to 0.9 μ and a width of from 0.3 to 0.5 μ for the small types. Larger forms of 0.6 by 1.4 μ were not uncommon and these dimensions are not maximum.

Colonial morphology was not distinctive, except for pigment formation. Colonies appear smooth, shiny, moist, convex, and with an entire edge. Pigment varied from a light or deep coffee brown to jet black, and appeared microscopically to be accumulated densely at the center with a clear narrow surrounding zone. Moist colonies could be lifted off entirely, and older colonies were drier and disintegrated when touched with a loop. Hemolysis on blood agar was noted in pure cultures four to seven days old, at a point where growth was heavy or confluent. In mixed culture there appeared to be more marked hemolysis. Under good conditions, colonies attained a diameter of several millimeters.

ISOLATION

Clinical material from which this organism may be obtained frequently is usually heavily loaded with a variety of organisms. As elsewhere reported, although initial growth is not difficult, isolation in pure culture is hazardous. Heart hormone agar, P_H 7.4, with 5 per cent defibrinated rabbit blood, as suggested by Burdon,[7] was satisfactory, as were other media. Freshly poured moist plates, inoculated immediately, were found to be most favorable. Slants, naturally moister, were used very successfully in rapid work. Plating technic, with repeated selection of colonies and transfer, was the only means found for isolation, although less time-consuming procedures were sought.

Other media on which growth was secured were:

1. Bordet-Gengou's medium.
2. Blood clot digest agar.
3. Veal infusion agar with 5 per cent rabbit or human blood; with human serum; with blood and 0.2 per cent cystine or 1 per cent glucose.
4. Brain broth with whole human blood; with human serum; with 30 per cent ascitic fluid.
5. Dehydrated (Difco) brain agar; with or without human whole blood or serum.
6. Extract ("Standard Methods") agar with rabbit or human blood, rabbit serum (P_H 6.4).
7. Liver hormone agar.
8. Semisolid (0.2 or 0.3 per cent) agar, with blood, serum, or ascites.
9. Loeffler's medium.
10. Beef heart broth with 0.05 or 0.1 c.c. whole blood per tube, or 30 per cent ascitic fluid; or without enrichment, with long established cultures.

No growth was secured with brain broth or veal broth or with dehydrated brain-heart broth (Difco) plus serum, blood, ascitic fluid, or adrenalin. Stimulating substances were not noted in experiments with autoclaved sputum, yeast filtrate, or carotin. In mixed cultures used in isolation a small amount of adrenalin[8] seemed to produce larger and moister colonies, although no effect was noted with pure cultures.

Anaerobic conditions were necessary for isolation and growth, although the organism did not appear to be an absolutely strict anaerobe. The choice of method appears to depend on convenience. Hydrogen replacement (Boez), phosphorus jars (Varney), deep shake tubes, Veillon tubes, Fortner plates, Buchner tubes with pyrogallol, semisolid agar, deep brain broth tubes (enriched), Wright's tubes (and Petroff's plates prepared as Wright's tubes), and Krumwiede's plate method, all were successfully used.

SPECIMENS YIELDING B. MELANINOGENICUM

The organism has been described as common in the normal mouth and external genitalia, and in pathologic material from mixed infections. Isolations were made in the course of this study from the mouth, from a number of cases of pulmonary abscess, from seven uterine swabs, from one psoas abscess, from two ischiorectal abscesses, from one parotid abscess, from one submaxillary abscess, from one abscess following tooth extraction, from one open mastoid, from one colpotomy, from one liver abscess following perforation of gastric carcinoma, from one empyema, from one gunshot wound perforating the stomach, from one open leg ulcer, and from one perineum. Pigmented colonies were recognizable usually after four to twelve days, with an average of six days and a maximum of twenty-one days of incubation.

GENERAL PHYSIOLOGY

The optimum temperature for growth appeared to be 37° C. Exposure to 60° C. for ten minutes and longer rendered a seventy-two-hour broth culture nonviable. Heavy inoculations were found to be advisable for transfer, particularly in shifting from solid to liquid medium. The organism sometimes developed slowly on primary growth, and thereafter required frequent trans-

fer until well established, as Burdon suggests. Intervals of six to ten days were successful, and viability was demonstrated after twenty-four days' incubation in semisolid blood agar; after thirty-two to thirty-seven days in brain 30 per cent ascitic fluid broth, but not after sixty days in the refrigerator; and after twenty-eight days but not after six weeks when kept at 37° C. on solid medium. A very disagreeable odor of putrefaction was noted, somewhat cheesy with solid media, and butyric with liquid and semisolid amount of gas was observed in pure cultures in brain ascitic fluid broth and media (enriched with ascitic fluid), in which the odor was greatest. A small in beef heart blood broth one to three days after inoculation.

Fermentation, with acid and gas, was observed, after overcoming some technical difficulties, with dextrose. Sodium phosphate extract broth[1] enriched with ascitic fluid or rabbit serum, proved satisfactory. Not all media allow fermentation. Maltose, levulose, and mannite occasionally yielded acid without gas; sucrose, lactose, dextrin, and galactose were never observed to break down.

Using ZoBell's method[9] no nitrate reduction was demonstrated; that is, nitrite tests were negative and nitrate tests remained positive. Indol tests were positive. Rather uniquely, brom cresol purple milk was acidified. It was also peptonized, as might be expected from proteolytic properties mentioned by Burdon. Slight liquefaction of Loeffler's medium was noted, partial digestion of brain particles in brain broth, and slight blackening of beef heart particles in beef heart broth after three or four days. Liquefaction of gelatin was not demonstrated although repeated tests were made, a discrepancy with reported results. Using brain 30 per cent ascitic broth, the liberation of H_2S was very marked.

PIGMENT PRODUCTION

The characteristic intense black pigment appeared to arise solely from blood substances. Repeatedly cultures produced pigment on blood medium, lost this property on medium without blood, although growing vigorously, and regained it when returned to a blood medium. In serum agar a late production of a reddish brown pigment appeared. Ascitic fluid and adrenalin appeared to be lacking in essential bases. Pigment appeared to be deposited after four or five days' growth (earliest noted, forty-two hours) and the development of a visible colony, first as a central brownish zone, later increasing to jet black and covering the whole colony. Bordet-Gengou's medium seemed especially favorable to pigment production, but no substance found chemically more definite than blood seemed to furnish the key to its development. When washed off with distilled water and dried on filter paper, it was found to be insoluble in distilled water, methyl alcohol, ether, chloroform, acetone, N/1 HCl, dilute ammonia; partially soluble in 10 per cent KOH (yellow solution, but pigment remained as granular masses), N/2 $NaCO_3$, and N/1 NaOH; soluble in acidified methyl alcohol (0.2 c.c. HCl to 5 c.c.) and in glacial acetic acid (pink color). Observations indicating that the pigment is toxic toward the organisms or that it is formed coincidentally with a toxic substance, were not convincing.

SEROLOGY

Extensive serologic tests were not considered advisable for the problem at hand. Agglutination tests, however, were performed with serum from rabbits given 11 injections intravenously in amounts of 1 to 4 c.c. of suspensions as high as 1 per cent. Antigen suspensions were made up to 0.25 per cent, preserved with Merthiolate. Agglutination set-ups were incubated two hours at 56° C., overnight at 37° C. and on ice for twenty-four hours. Control checks remained negative, and titers reached 1:1280 with +++ reactions, with some agglutination at 1:10,240 with one serum. Clumping was granular rather than flocculant. In support of this definite agglutination reaction a miscellaneous collection of strains was brought together and tested against specific antiserum. Although the titers were not equally high with all strains, there was no reason to postulate the existence of a wide variety of serologically heterogeneous strains.

PATHOGENICITY

B. melaninogenicum was used, both in pure cultures and in mixed cultures, in experimental work on pulmonary abscess, the results of which will be reported elsewhere (Stevens and Shevky). No evidence was found that this organism had any invasive capacity, however, which is consistent with the observations mentioned herein. Burdon found[7] with one strain an extensive cutaneous gangrene following subcutaneous inoculation, without septicemia, but with death in forty-eight hours. Most of the meager evidence available indicates a lack of pathogenicity.

Control checks were not extensive. Three each of rabbits, guinea pigs, rats, and mice were cultured around the gingival margins, and *B. melaninogenicum* was found in one rabbit. Bacteriologic studies of experimentally inoculated animals did not lead one to suspect that the organism was particularly common as a commensal in local stock animals.

Attempts to produce evidence of necrotizing properties by intracutaneous inoculation resulted in very minor or negative reactions. A suspension of organisms washed from a blood agar slant incubated fourteen days was matched with a 0.25 per cent typhoid bacillus antigen. The culture used originated from pulmonary abscess material seven weeks previously. Two series of three animals each of rabbits, guinea pigs, rats, and mice were used. Saline washings from blood agar slants were used as controls. The inoculum was 0.1 c.c. Consistent observation revealed several slight nodules or slight inflammation, but even these were insignificantly more marked than control reactions.

Attempts to induce invasion by intraperitoneal injection were performed with cultures from five to eight days old. Two series of 12 animals each, as in intracutaneous experiments, were used. Both blood agar and brain ascitic fluid broth cultures were used, with heavy inoculations (rabbits 5 and 10 c.c.; guinea pigs 3 and 5 c.c.; rats 3 c.c.; mice 1 c.c.) of standard suspension. Emulsions heated at 60° C. for two hours were used as controls. Observation was carried up to forty days. There appeared to be no evidence of

any kind, symptomatic, pathologic, or bacteriologic, of localization and multiplication of the organism. Since one series was carried through with densely pigmented growth and the other (broth culture) with no evidence of pigment, this property did not seem to be a factor in pathogenicity.

Intravenous inoculation of rabbits with large doses of both young and old cultures, variously made up, resulted in no abnormal temperatures or loss of weight, and the animals were continued with large repeated doses to develop an antiserum without apparent effect on their health. After final bleeding, autopsy revealed no significant changes, and cultures from the knee joint, the shoulder joint, right and left lobes of the lungs, liver, and spleen remained negative.

Attempts were made to introduce the organism directly into pulmonary tissue. Suspensions of young cultures were used, matched to both 1 and 2 per cent typhoid antigen. Quantities of 0.1 c.c. (rabbit), 0.08 c.c. (guinea pig), and 0.04 c.c. (mouse) were used in each series. The animals gained in weight and showed no abnormal temperatures during a month of observation. Autopsy revealed normal organs and negative cultures.

Intracerebral inoculations were given two series of animals, three guinea pigs and seven mice in the first, and four guinea pigs and two mice in the second. The animals were young, and several strains of young cultures were tried. Although maximum doses were used, there was no sign of any reaction whatever in any animal during a twenty-one-day period of observation. One mouse sacrificed in forty-eight hours yielded negative brain cultures.

SUMMARY

B. melaninogenicum appears to be common as a commensal and on surfaces normally or pathologically having a mixed flora. Detailed study suggests that this organism conforms to single type and is not one of a group of biochemically, morphologically, or serologically different types. All attempts, however, to show that it had any significance as a primary or secondary invader in mixed infections gave negative results, so far as clinical evidence or attempts in experimental animal infection could be correlated.

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THE TREATMENT OF RHEUMATIC CARDITIS WITH AQUEOUS EXTRACTS OF STREPTOCOCCI*

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RHEUMATIC fever has received a great deal of attention during the past decade but the problems of its specific etiology, prophylaxis, and treatment have scarcely advanced beyond the stage of hypothesis. This serves to establish special interest in the disease aside from that engendered by the vastness of its humanitarian, social, and industrial aspects. One theory regarding its specific etiology is, that streptococci are in some manner concerned as the causative agents. Concerning the manner in which they act, and the strain or strains which are responsible, there is a wide divergence of opinion.

This discussion of the treatment of rheumatic carditis will be limited to the use of aqueous extracts of streptococci administered by injection after the manner of vaccines. It will introduce a relatively new system of dosage of bacterial products which has been developed during the past six years by means of applying aqueous extracts of streptococci in the treatment of patients suffering from chronic atrophic arthritis.

The relationship between rheumatic fever and atrophic or rheumatoid arthritis is aside from this discussion. All that concerns it is, that both groups of patients present at least one feature in common. This is a marked reactivity, or hypersensitiveness to streptococci, or to their derivatives administered parenterally. This character has been designated "hyperergy" by Swift.[1] It is an outstanding feature of the "rheumatic state." Many types of streptococci have been used in demonstrating the existence of this hypersensitiveness, so that the type of streptococcus used in studying the reactions arising in patients appears to be less important than does the fact that it characterizes patients both with rheumatic fever and with rheumatoid arthritis.

The reactions excited in arthritis by the injection of extracts of streptococci[2] furnish an almost ideal means of studying the degree of this hypersensitiveness. An outstanding feature of the reactions in arthritis is an activation of lesions in the joints which are so superficially distributed as to permit direct observation of objective symptoms. Trial dosage if it produces a severe reaction does not endanger life even though it may increase considerably the patient's discomfort. On the other hand the patient with rheumatic fever presents reactions in the heart as well as in the joints, and for this reason cannot safely be treated with overdosage. After dosage and certain principles in the treatment of the hypersensitiveness of atrophic arthritis had been established, it was a relatively simple matter to apply them in the treatment of similar hypersensitiveness characterizing rheumatic carditis.

*Received for publication, April 15, 1933.

DOSAGE OF THE EXTRACTS OF STREPTOCOCCI

The degree of this hypersensitiveness of the "rheumatic state" was determined by responses of patients with atropic arthritis to minute doses of aqueous extracts of streptococci. Throughout this study a saline extract of streptococcus cardioarthritidis was used under the designation of a soluble antigen.*

This product is prepared by suspending the streptococci in normal saline as in a vaccine. The concentration is relatively weak, being 100 millions per cubic centimeter. After seven days in a refrigerator, the bacteria are removed by filtration. The water clear menstruum containing the extractives is the undiluted soluble antigen. A series of sixteen dilutions of this are made, each successive one being one-tenth the strength of the last. The dilutions are conveniently designated as follows: 10^{-1}; 10^{-2}; 10^{-3} and so on up to 10^{-16}. A series of twelve dilutions only, beginning with 10^{-5}, and ending with 10^{-16} is used in treatment of arthritis, but only the last three, namely, 10^{-14}; 10^{-15}; and 10^{-16} are suggested in rheumatic carditis.

CHARACTERS OF THE REACTIONS

The reactions excited in this form of therapy are nothing more than aggravations of existing symptoms. They do not introduce new symptoms such as chills, high temperatures, etc., as are common in foreign protein therapy and which appear less frequently with the customary large doses of vaccines. They are triphasic but the triphasic character is brought out only by moderate overdosage. These three phases have been designated[2] "primary reaction," "period of euphoria" and "secondary reaction." They have also been observed by Crowe[3] in his work with small doses of vaccine. He calls them "reaction," "response," and "relapse."

Proper dosage for the patient is followed by a period of euphoria beginning immediately upon the absorption of the antigen and lasting for a variable period of days (Chart 1). The outstanding features of this are relief of pain, buoyancy of spirits, and general stimulation. The euphoria terminates in the secondary reaction or "relapse," as Warren Crowe has more aptly named it. Administration of another suitable dose during the secondary reaction promptly terminates it by instituting another period of euphoria.

The primary reaction with moderate overdosage appears within the first twenty-four to forty-eight hours. It is usually followed by a period of euphoria which in turn terminates in the secondary reaction. It is a curious fact that further increases up to what may be termed gross overdosage do not proportionately increase the severity of the symptoms but rather tend to produce less stormy primary reactions which are delayed in their onset and prolonged in their course. These are referred to as "delayed primary reactions." They are not followed by periods of euphoria.

The delayed primary reaction begins forty-eight hours or more after an injection. During this interval no change is noted in the patient's clinical

*The soluble antigen used in this study has been supplied through the courtesy of Sharpe and Dohme, Philadelphia, Pa.

condition. It comes on gradually with symptoms which are of a general character rather than focal; e.g., malaise, lassitude, and generalized aching. It tends to be prolonged. Administration of another injection maintains the symptoms.

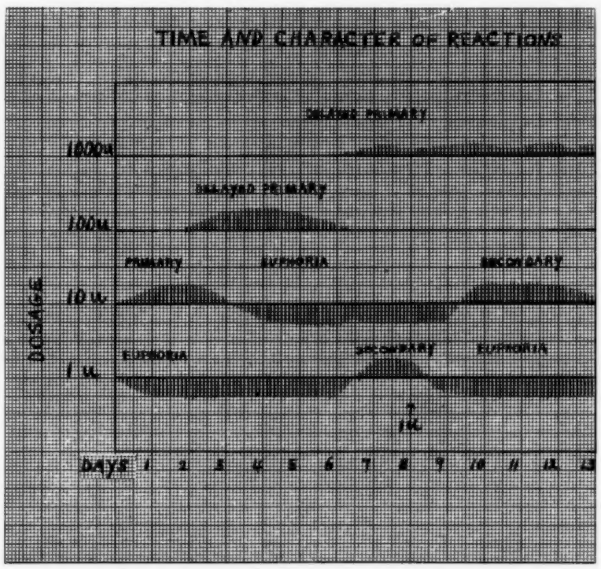

Chart 1.—In this diagrammatic chart the average level of the patient's symptoms is indicated by the solid lines at levels *1 u, 10 u, 100 u* and *1000 u.* The swing of the symptoms above or below this level is represented by the hatched lines. Suitable, or unit dosage of the extractives of streptococci is indicated at *1 u,* and excessive dosages at *10 u, 100 u* and *1000 u.* The different reaction phases and their relations to the time of injection are illustrated.

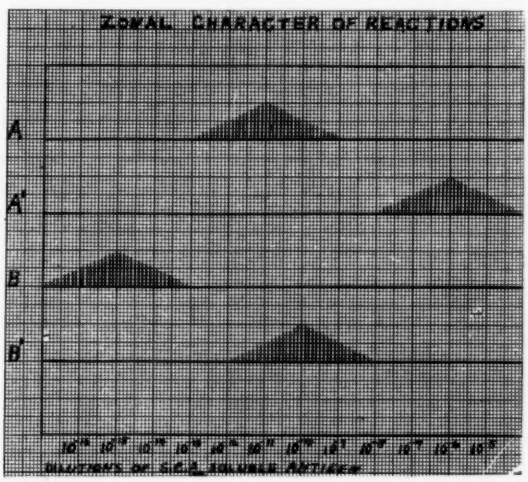

Chart 2.—At levels *A* and *B* are represented the zones of reaction of different patients following injections of the extractives of streptococci. At levels *A'* and *B'* the shifting of these zones to ranges of stronger dilutions is shown.

The secondary reaction follows a period of euphoria, usually of more than forty-eight hours' duration, during which there had been a well-marked improvement in the patient's clinical condition. It comes on abruptly with symptoms which are largely focal in type; e.g., increased pain, swelling, and red-

ness about the involved joints. It is terminated promptly by repeating the injection of the antigen.

ZONAL CHARACTER REACTIONS

The reactions are limited to a certain definite range of dosage. The administration of amounts below or above this range is not followed by reactions. This range varies for different individuals, and it shifts to stronger dilutions as treatment is continued in a given patient (Chart 2). This zonal character of the reactions is a very unusual phenomenon. Its recognition has great practical importance in adjusting dosage as will appear later under the discussion of the principles in the applications of treatment.

DISCUSSION

The extreme dilution of these extracts at first appears to be an absurdity. Experience over the period of years, during which repeated checks with blank solutions carrying only the saline and the traces of preservative used in extracts, have shown that contact with the streptococci imparts something not present in the blanks, which is potent in inducing reactions in arthritis even in these dilutions. The dilutions were arrived at gradually through trials of them in arthritis proceeding from the stronger ones down through the weaker ones. The guiding purpose was that of obtaining dilutions so weak that when convenient amounts were administered to patients with arthritis, no activation of the joints would follow. Encouragement was had from the fact that as the reactions were avoided, clinical improvement became more constant and more pronounced.

Another advantage of these minute dosages developed as the work progressed. They appear to act in the direction of desensitization only, perhaps because they are too small to increase the patient's reactivity. There is no need therefore to resort to intravenous injection as a precaution against this. This is a decided advantage over vaccines which even in small doses are prone when injected subcutaneously to reverse the process thereby increasing the patient's reactivity.

These doses are smaller than any yet used in biologic therapy. The nearest approach to them are those of tuberculin therapy. Others have used small doses of vaccines of streptococci in treatment of arthritis, but they can scarcely be compared with the ones used in this work. Warren Crowe,[3] who has been using polyvalent vaccines of streptococci in arthritis for some twenty years, has arrived at a minimal dosage of 100 (only) microorganisms, and seldom goes above a dosage of 10 millions. Burbank[4] states that some patients require initial doses of 50 (only) bacteria of polyvalent streptococcus vaccines. Each of these workers as experience was gained, has been reducing dosage.

PRINCIPLES IN THE APPLICATION OF THIS THERAPY

In contrast with treatment of foreign protein "shock" therapy, reactions must be avoided in this therapy if results are to be obtained. At all times to

avoid doses which are followed by reactions becomes the first principle of treatment. Since dosage may be above as well as below that range in which reactions occur, this principle must be further qualified by stating that dosage must be *below* that followed by reactions. This can be determined only by trial in the individual case, beginning with the weakest dilutions and gradually increasing dosage until a mild reaction appears. Thereafter dosage should be decreased slightly for best results. The continued use of doses within the zone of reactions aggravates the patient's symptoms. Persistent dosage above this zone, within certain limits, appears to influence the disease very little one way or the other.

The second principle is, that dosage to be most effective should be followed by periods of euphoria. It is not sufficient to avoid reactions but improvement must be obtained upon the absorption of the material injected. The optimum dosage is that amount which is followed by the longest period of euphoria. An amount which is followed by a period of euphoria lasting more than five days is regarded as satisfactory early in a course of treatment. If the euphoric periods are shorter than five days the dosage should be increased fractionally. The periods of euphoria tend to grow shorter under constant dosage during a course of treatment. When this occurs, it constitutes an indication for increasing dosage.

A third principle may be stated. Increase dosage only when the repetition of fixed amounts is followed by a shortening of the periods of euphoria. The periods of euphoria terminate in the secondary reaction. The appearance of this latter indicates that more antigen must be administered since the benefits derived from the last injection are at an end. Later in the course of treatment, as dosage is gradually built up the periods of euphoria lengthen, and secondary reactions grow milder. Finally the secondary reactions fail to appear as the arthritic process becomes quiescent.

The chief difficulty arising in practice is that of differentiating the delayed primary reaction of gross overdosage from the secondary or "relapse" reaction which calls for a repetition of the dose. One must rely on the symptoms presented in attempting to differentiate between the two reactions. If uncertain, the course of events following a repetition of the dosage usually suffices to clear up the doubt. Relief follows the repetition of dosage in the case of a secondary reaction and fails to do so in case of the delayed primary reaction.

PRESENTATION OF SELECTED CASES OF RHEUMATIC CARDITIS

With this information fairly well correlated and upon a background of experience in favorably influencing the "rheumatic state" as evidenced in arthritis, the problem of influencing in a similar manner the "rheumatic state" as seen in rheumatic fever has been approached by practically identical methods of practice. To present this work, a small group of 13 patients has been selected. These were chosen not because they demonstrate the most favorable results with this therapy but rather to illustrate certain errors which were made because of premature attempts to apply the method in rheumatic

conditions during the course of its development in arthritis. Thereby the parallelism between its applications in the two conditions can best be demonstrated. More recently all except one of these have received the benefits of dosage more properly adjusted to their needs. The observations on ten of them have extended over periods ranging from two to more than five years. The remaining three have been observed for less than a year, and serve to illustrate the results with the present methods. In three patients acute exacerbations followed injections of the larger doses which were employed from two and a half to four years ago. These followed an injection closely enough to suggest that they represented the primary type of reaction. Acute exacerbations have not occurred with dilutions of the soluble antigen weaker than 10^{-11}. They have also been absent as a spontaneous occurrence in patients treated by the weaker dilutions during the past two and a half years. In Table I are presented the summary of the outstanding clinical manifestations, the range of dosage used, and the results in each of the thirteen patients. Four (J. G., R. H., J. J., and T. K.) were seen first in acute attacks of rheumatic fever, or chorea, and treatment with the soluble antigen of streptococcus cardioarthritidis was begun with the passing of the acute symptoms. Six (E. H., K. N., S. W., W. R., A. L., and J. B.) were seen first during convalescence from a febrile attack of rheumatic fever or chorea. Three (R. McC., M. C., and M. L.) presented long-standing rheumatic disease of the heart, which was still active. Two of these had had no acute attacks of rheumatic fever or chorea. The other had two attacks of chorea and three of rheumatic fever. Only two patients were seen in their initial attack of rheumatic fever. The others, with the exceptions mentioned above, had had multiple attacks of rheumatic fever, or chorea. Eight of the patients were children with ages ranging from three years to thirteen. Three patients had active chorea when first seen, two had subcutaneous nodules, and another had a history of them with a previous attack of rheumatic fever. Three had pericarditis. One had acute iritis accompanying rheumatic fever with pancarditis. All had valvular cardiac lesions.

The range of dosage of the soluble antigen of streptococcus cardioarthritidis is charted opposite each patient's record in Table I. It will be seen that in the earlier applications of this antigen the doses were larger and that later ones were smaller. Three patients developed acute exacerbations and four others exhibited mild reactions to individual injections. All of these were among the earlier cases and all with the larger doses employed at the time. One of these patients died of cardiac failure in an acute exacerbation of pancarditis, the remaining six patients responded favorably when doses were reduced below the zone of reactions and have remained quiescent since.

The case (J. G.) is of interest because only mild reactions followed large individual doses, and the acute exacerbation occurred while dosage was being reduced. It represents an instance of tolerance to large doses and the entering of the zone of reaction from above. The other instances of acute exacerbations and of mild reactions to individual doses occurred while dosage was being increased. They are examples of entering the zone of reaction from below.

TABLE I. THE RANGE OF DOSAGES USED IN THE TREATMENT OF EACH PATIENT IS CHARTED

The range of dosages to which no reactions occurred is indicated by straight lines. The wavy lines show the range of dosages to which mild reactions occurred. Acute exacerbations are indicated by the hatched lines.

RHEUMATIC CARDITIS

Clinical Summary of Patients Treated.

RANGE OF DOSAGE OF S.C.A. SOLUBLE ANTIGEN

	10^{-16}	10^{-15}	10^{-14}	10^{-13}	10^{-12}	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	Age	Sex	Date	Diagnosis	Previous Attacks	Present Condition
JG													8	M	6-21-27	R.F.; Pan. card. Subcut. nod.		Died 2/25/29
RH													21	M	5-24-27	R.F.; Aortic and Mit. regurg.	Two of R. F.	Quiesc. Aortic Mitral
E H													26	F	9-27-27	R.F.;Pancard. Iritis; Subc.nod.	R. F.at 24 yrs. R.F.;Pericard.at 25	" Mitral
RMC													24	M	6-18-28	R.Card.Mitral and aortic	Chorea @ 6 & 10 yrs R.F. @ 18,19,& 24	" Mitral Aortic
JJ													11	F	6-22-30 8- 1-32	Chorea; Chorea; Mit. Sten.	Chorea @ 10 yrs.Per sist.10 & 11th yrs.	" Mitral
KN													11	F	8-11-30	R.F.;Pericard. and carditis	R.F. @ 9 & 10 yrs.	" Mitral
SW													3	M	5-27-29	R.F.; Aort. & Mitral	None	" Mitral
TK													4	M	6-20-29	R.F.;Mit. regurg.	None	" Mitral
WR													11	M	6-24-30 11- 6-31	R.F.;Mit.regurg. Mit. stenosis	R.F.@ 6 yrs.Recur. mild feb. attacks	" Mit. & Aort Cor.bovinum
MC													36	F	11- 1-30	R.card.;Aort.regur. Mitral stenosis.	Tonsil.& growing pains only	" Mitral & Aortic
AL													13	F	1-22-32	R. F.; Mitral regurg.	R.F.@ 7 yrs; Tons. & grow.pains since	" Mitral
JB													12	F	3-28-32	Chorea; Aortic (?) & Mit. regurg.	Chorea @ 9yrs;R.F. & subc.nod.@10 yrs	" Mitral
ML													43	F	9- 8-32	R.card.;sciatica Mit. stenosis	R.heart @ 12 yrs Decomp.@ 28 yrs	" Mitral

KEY OF SYMBOLS:— ZONE OF NO REACTIONS.
ZONE OF MILD REACTIONS.
ACUTE EXACERBATIONS.

Patient J. J. is also worthy of special note since she was treated by repeated courses of injections of dilutions 10^{-8} and 10^{-9} during a period of two years without noteworthy effect on a persistent chorea of low grade. When dosage was dropped to the 10^{-16} and 10^{-15} range, the choreic symptoms disappeared and improvement in the general condition became evident after a few weeks.

In order to present more in detail the methods of this treatment Tables II and III are included.

TABLE II

K. N., AGED ELEVEN YEARS, AUG. 11, 1930. DIAGNOSIS: CONV. RHEUMATIC FEVER AND PERICARDITIS, RHEUMATIC CARDITIS, MITRAL REGURGITATION

DATE	WT.	PULSE	HG	W.B.C.	SED. R.B.C.	OP. IND.	DOSAGE S.C.A. ANT.	REMARKS
8/11/30	57	120	70	10,700	36	0.9	0.1 10^{-12}	Treated in office. Rest in bed all day
8/18/30	60	92					0.3	
8/28/30	65¾	90					0.1 10^{-11}	
9/ 4/30	67¾	90					0.3	On 9/9/ relapse. Temp. 103°;
9/12/30		105					0.1	pulse 102; dil. heart. 9/13/
9/17/30		105					0.1	norm. temp.; much improved
9/24/30		90					0.1	Confined to bed
10/ 2/30		90				0.8	0.1	
10/ 9/30	69½	84					0.1	Patient returns to office
10/16/30	70½	80	70		12	0.7	0.1 10^{-12}	
10/23/30	71¾	84					0.1	Rest in bed greater part of day
10/30/30	73	96	70		13	0.7	0.1 10^{-14}	
11/ 6/30	74½	80					0.1	
11/13/30	73	96	70		14	0.8	0.05	
11/20/30	72¼	92					0.05	Out of bed. Restricted activities
11/26/30	72¼	74	80		27	1.0	0.05	
12/ 4/30	73½	74					0.05	
12/11/30	75¾	78			18	0.7	0.05	
12/18/30	76½	78	80	12,000		0.7	0.05	
12/29/30	78	72		9,500		1.1	0.1	
1/ 8/31	78¼	81				0.5	0.1	
1/15/31	78¼	78					0.1	
1/22/31	79½	84					0.1	
1/29/31	79¾	78	65		11	0.9	0.1	Returned to school second half year. Week interval and dosage continued unchanged
4/11/31	80½	78	80		12	1.6	0.1	Week interval and dosage advanced to 0.15 c.c. 10^{-14}
5/16/31	81¼	78	80		15	0.9	0.15	14 day interval began dosage 0.15 c.c. 10^{-16}
7/ 7/31	82½	78		7,300		0.8	0.2	21 day interval dosage 2 to 2.5 c.c. 10^{-14}
1/ 8/32	86½	72			8	1.3	0.3	28 day interval dosage 0.3 c.c. 10^{-14}
11/ 4/32	101½	72			12	1.5	0.4	Dosage increased
12/ 9/32	104	72					0.5	Dosage increased

In Table II are tabulated the dosage, intervals between treatments, certain clinical and laboratory data in the case of K. N. who showed a relapse under treatment when dosages were being increased rapidly. While a gain in body weight and a decrease in pulse rate occurred from the first, a definite tendency to quiescenec of the rheumatic carditis did not appear until the

dosage was dropped from 10^{-12} dilution to 10^{-14} dilution. Amounts of 0.05 c.c. to 0.1 c.c. of the latter were, in the light of further experience, still too large so that not until after treatment had been carried out for about five months was progress regarded as satisfactory. Apparently by this time a tolerance to a dosage of 0.1 c.c. of dilution 10^{-14} had been established. This long pre-

TABLE III

W. R., AGED ELEVEN YEARS, NOV. 6, 1931. DIAGNOSIS: RHEUMATIC CARDITIS, AORTIC REGURGITATION, MITRAL REGURGITATION

DATE	WEIGHT	PULSE RATE	HG	SED. R.B.C.	DOSAGE 10^{-15}	REMARKS
11/ 7/31	84½	108	73	35 mm.	0.05	Treatment in office. Activities greatly restricted. W.B.C. 9,800 Opsonic index 1.0
11/13/31	85	112			0.1	
11/20/31	84½	96	75	26 mm.	0.1	
11/27/31	88	96			0.1	
12/ 4/31	89½	100			0.15	
12/11/31	89	96			0.15	
12/18/31	87½	96			0.1	
12/28/31	89½	96	88	16 mm.	0.1	
1/ 9/32	92	96			0.1	Return to school where use of elevator was arranged
1/23/32	94	96			0.1	
2/ 6/32	93	88			0.1	
2/20/32	95¾	90			0.1	
3/ 5/32	96½	96		10 mm.	0.1	
3/26/32	94¾	105			0.1	
4/16/32	97½	92			0.12	
5/14/32	97½	84	98		0.15	
6/11/32	100½	90			0.2	
7/ 6/32	98¾	84		18 mm.	0.15	Severe cold
7/29/32	98¼	84			0.15	
9/ 9/32	104¼	96		7 mm.	0.1	After one month at shore
10/15/32	106¾	90			0.15	
11/11/32	108¾	84			0.2	
12/ 9/32	110	84	88	8 mm.	0.2	Grippe 12/24 W.B.C. 8,400 Opsonic index 1.0
1/ 6/33	108¾	78			0.2	

liminary period is not observed in cases where treatment is begun with a much smaller dosage. This is illustrated in the record of patient W. R. as presented in Table III.

It is desired to call attention to the intervals between treatments as employed in these patients. Treatment is begun at seven-day intervals. After a course of seven or more injections, intervals of ten days are employed. Later fourteen, twenty-one, and twenty-eight days are allowed to elapse between treatments.

The treatments at monthly intervals are continued for at least a year after the carditis appears quiescent by all criteria used in estimating its activity. This is a precaution against relapse in a disease of the persistent character amply demonstrated for rheumatic carditis. When treatment is stopped, it is discontinued preferably during the summer months or at a season of lowest prevalence of the acute manifestations of rheumatic fever. In addition to the clinical observations used in estimating quiescence or ac-

tivity of rheumatic carditis, laboratory determinations of the hemoglobin, of erythrocytes and leucocytes, and of sedimentation distance of erythrocytes are used in following the course of the disease. Since the extract employed has been prepared from a single strain of streptococcus cardioarthritidis, the opsonic index readings of the patient's serum against this strain have been found to be most helpful in the matter of regulating dosage. Overdosage is regularly reflected in its depression of the opsonic index.

PRACTICAL APPLICATION IN RHEUMATIC CARDITIS

The treatment of rheumatic carditis with the aqueous extract of streptococcus cardioarthritidis as developed may be briefly outlined. An initial dosage of 0.05 c.c. of dilution 10^{-16} of the soluble antigen is injected subcutaneously. The injections are repeated at intervals of from five to seven days, using 0.05 c.c. each for the first three injections. If tachycardia, cardiac arrhythmia or mild rheumatic pains develop within the first forty-eight hours the amount must not be increased until these symptoms fail to appear following an injection. Therefore limit any increase of dosage to 50 per cent, or less of the amount last administered. The most favorable results are obtained when the dosage is adjusted so as to avoid reactions. Improvement manifests itself in the erythrocyte count, leucocyte count, the hemoglobin, the sedimentation distance of erythrocytes, and the opsonic index of the patient's serum against streptococcus cardioarthritidis after from four to six treatments. No improvement usually indicates too large a dosage of the antigen.

Evidence of continued improvement after six or eight treatments at intervals of from five to seven days should be followed by treatments at intervals of from ten to fourteen days. Later the intervals may be lengthened to three or four weeks. Treatments at monthly intervals should be continued for at least a year after all signs of activity have disappeared. Dosage begun at 0.05 c.c. of dilution 10^{-16} is usually not carried above 0.1 c.c. of dilution 10^{-14}. Dilutions stronger than 10^{-14} are not regarded as necessary and are not recommended in rheumatic carditis.

SUMMARY

There is a range of dosage of the aqueous extracts of streptococcus cardioarthritidis in which dangerous exacerbations of rheumatic fever occur.

In the patients observed to date such exacerbations have not occurred with a range of dilutions weaker than that designated dilution 10^{-11} streptococcus cardioarthritidis soluble antigen, and mild reactions to individual doses occur infrequently in the range of dilutions weaker than dilution 10^{-13}.

When dosages have been kept below those amounts followed by mild reactions to the individual injections clinical improvement has occurred regularly.

Dilutions 10^{-16}, 10^{-15}, and 10^{-14} only are recommended in the treatment of rheumatic carditis.

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THE LIGHT FILTERING INDEX OF BLOOD SERUM*

A Discussion of Its Clinical Application

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IN A PREVIOUS paper,[1] it was suggested that the power of human blood serum to filter out light might possibly be utilized as a prognostic aid. The present paper records a technic which renders this suggestion practical, and presents a discussion of the procedure as applied to medical conditions.

Inasmuch as both the method of examination and the theoretical basis upon which the procedure under discussion is founded are somewhat novel, it seems advisable to consider certain aspects of the matter in detail. We have to deal with two factors: (a) serum and (b) light. Viewed from the purely physical standpoint, serum is a colloidal system. If one be permitted to revert to an elementary presentation, a colloidal system may be said to be composed of particles which may, or may not, vary in size, and which are separated from one another by varying amounts of a substance of different physical characteristics than the aforementioned particles, this separating substance being termed interface. Picturing such an arrangement one may conceive that a light wave passing through the serum encounters the colloidal particles, and that these either block, absorb, or reflect the light, thus acting as a filter. The amount of light which is held back would vary according to the conditions within the colloid system, and these conditions are dependent upon the size of the particles, the amount and nature of the interface, and the arrangement of particles and interface.

The current concept of light is that it is energy in the form of a wave and that the color of the light is related to, or dependent upon, the length of the wave. The unit by which the length of the light wave is measured is the Ångström's unit which is one ten-millionth of a millimeter.

*From the Achelis Laboratory, Lenox Hill Hospital.
Received for publication, May 12, 1933.
Under a grant from the Fred Weingarten Fund.

tivity of rheumatic carditis, laboratory determinations of the hemoglobin, of erythrocytes and leucocytes, and of sedimentation distance of erythrocytes are used in following the course of the disease. Since the extract employed has been prepared from a single strain of streptococcus cardioarthritidis, the opsonic index readings of the patient's serum against this strain have been found to be most helpful in the matter of regulating dosage. Overdosage is regularly reflected in its depression of the opsonic index.

PRACTICAL APPLICATION IN RHEUMATIC CARDITIS

The treatment of rheumatic carditis with the aqueous extract of streptococcus cardioarthritidis as developed may be briefly outlined. An initial dosage of 0.05 c.c. of dilution 10^{-16} of the soluble antigen is injected subcutaneously. The injections are repeated at intervals of from five to seven days, using 0.05 c.c. each for the first three injections. If tachycardia, cardiac arrhythmia or mild rheumatic pains develop within the first forty-eight hours the amount must not be increased until these symptoms fail to appear following an injection. Therefore limit any increase of dosage to 50 per cent, or less of the amount last administered. The most favorable results are obtained when the dosage is adjusted so as to avoid reactions. Improvement manifests itself in the erythrocyte count, leucocyte count, the hemoglobin, the sedimentation distance of erythrocytes, and the opsonic index of the patient's serum against streptococcus cardioarthritidis after from four to six treatments. No improvement usually indicates too large a dosage of the antigen.

Evidence of continued improvement after six or eight treatments at intervals of from five to seven days should be followed by treatments at intervals of from ten to fourteen days. Later the intervals may be lengthened to three or four weeks. Treatments at monthly intervals should be continued for at least a year after all signs of activity have disappeared. Dosage begun at 0.05 c.c. of dilution 10^{-16} is usually not carried above 0.1 c.c. of dilution 10^{-14}. Dilutions stronger than 10^{-14} are not regarded as necessary and are not recommended in rheumatic carditis.

SUMMARY

There is a range of dosage of the aqueous extracts of streptococcus cardioarthritidis in which dangerous exacerbations of rheumatic fever occur.

In the patients observed to date such exacerbations have not occurred with a range of dilutions weaker than that designated dilution 10^{-11} streptococcus cardioarthritidis soluble antigen, and mild reactions to individual doses occur infrequently in the range of dilutions weaker than dilution 10^{-13}.

When dosages have been kept below those amounts followed by mild reactions to the individual injections clinical improvement has occurred regularly.

Dilutions 10^{-16}, 10^{-15}, and 10^{-14} only are recommended in the treatment of rheumatic carditis.

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THE LIGHT FILTERING INDEX OF BLOOD SERUM*

A DISCUSSION OF ITS CLINICAL APPLICATION

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IN A PREVIOUS paper,[1] it was suggested that the power of human blood serum to filter out light might possibly be utilized as a prognostic aid. The present paper records a technic which renders this suggestion practical, and presents a discussion of the procedure as applied to medical conditions.

Inasmuch as both the method of examination and the theoretical basis upon which the procedure under discussion is founded are somewhat novel, it seems advisable to consider certain aspects of the matter in detail. We have to deal with two factors: (a) serum and (b) light. Viewed from the purely physical standpoint, serum is a colloidal system. If one be permitted to revert to an elementary presentation, a colloidal system may be said to be composed of particles which may, or may not, vary in size, and which are separated from one another by varying amounts of a substance of different physical characteristics than the aforementioned particles, this separating substance being termed interface. Picturing such an arrangement one may conceive that a light wave passing through the serum encounters the colloidal particles, and that these either block, absorb, or reflect the light, thus acting as a filter. The amount of light which is held back would vary according to the conditions within the colloid system, and these conditions are dependent upon the size of the particles, the amount and nature of the interface, and the arrangement of particles and interface.

The current concept of light is that it is energy in the form of a wave and that the color of the light is related to, or dependent upon, the length of the wave. The unit by which the length of the light wave is measured is the Ångström's unit which is one ten-millionth of a millimeter.

*From the Achelis Laboratory, Lenox Hill Hospital.
Received for publication, May 12, 1933.
Under a grant from the Fred Weingarten Fund.

It has been found by actual observation (1) that light of different wave lengths, when passed through a layer of serum of definite thickness, the intensity of the light being relatively constant, is held back in varying degrees. It is upon this observation that the method of examination is based.

The Pulfrich photometer equipped with two filters having as their center of light filtration the following wave lengths (plus or minus 100 Å units) is employed, 7,200 Å and 4,300 Å. The mechanism and operation of this instrument has been described[1] previously. A standardized cell having a depth (thickness of serum layer) of 2.5 mm. is used and this cell is so constructed that the width of the serum layer is 3 mm. The portions of the cell which are not actually the walls of the cell cavity itself are painted black to cut out extraneous light. Blood for examination is collected in a Wright capsule after finger puncture. To give a sufficient amount of serum the body of the capsule should be 3.5 cm. in length and the bore approximately 8 mm. Blood can also be obtained by venepuncture, but this method is not necessary. Hemolyzed sera should be discarded. The time of taking the specimen is not material, if not too soon after eating, provided that in any given instance it is taken at the same time in all subsequent examinations; for example, if the first determination is made on a specimen collected before breakfast or two hours after breakfast, then subsequent specimens should be collected at the same relative time of day. After the serum is separated from the clot it may be examined at any time within twelve hours.

The standardized cells of the photometer are filled, one with distilled water, the other with the serum to be examined. In the manner described in the booklet of instructions which accompanies the instrument, the amount of light which is passed by the serum with each of the specified filters is then determined. The reading obtained with the 4,300 Å filter is subtracted from that obtained with the 7,200 Å filter. The result thus obtained indicates the amount of light, of the band situated between the central points of the two chosen filters, which has been blocked out. This result is then divided by the number of 100 Å units present in the band; i.e., 29.

Example.—The reading with the 7,200 filter indicates the passage of 95 per cent of the light, while with the 4,300 filter, 40 per cent of the light is passed. Between these two filters then, 55 per cent of the available light has been lost (95 minus 40). Since in the band between 7,200 and 4,300 there are 2,900 units, 55 is divided by 29 giving a quotient of 1.89. The filtration index in this instance is 1.89, but since in the computations recorded in this paper the second decimal place is either advanced one if above 5 or discarded if below 5, the corrected index is 1.9.

Expressed concretely, the light filtration index is obtained by subtracting the amount of light passed by the filter with the shorter wave length from that passed by the filter with the longer wave length, and dividing the result by the number of 100 Å units in the band lying between the center points of the two filters.

Isolated determinations of the index are valueless both from the diagnostic and prognostic standpoint, a question which has been considered in

the first report. It is only if the index is determined daily, or at possibly slightly less frequent intervals, that the collected data become of value. In the present paper we shall consider the application of the light filtration index in a group of medical conditions, surgical conditions being considered in another paper by Dujat and Kahan. Based upon 129 single observations made on normal individuals, the light filtration index normally varies between 1 and 3.1 with an average of 2.1. The distribution of the varying indices over the range, as expressed in percentages of the group is shown graphically in Chart 1.

A series of six normal individuals were examined every other day for a period of fifteen days. The data of these cases is given in Table I. In Case 1 the index was constant over the entire period. In Case 2 the index was constant over the entire period, except for the seventh day, the drop of 0.1 being

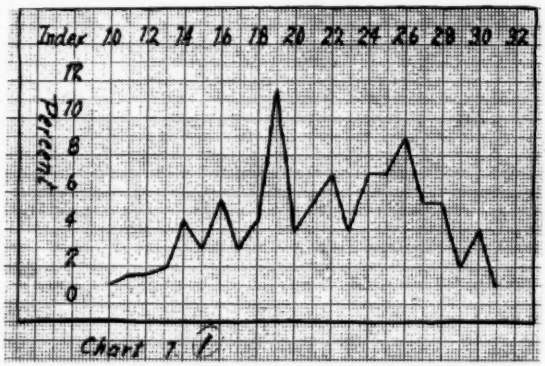

Chart 1

associated with constipation. In Case 3 the minor variations were associated with menstruation. In Cases 4, 5, and 6 the changes in the index were associated with constipation.

TABLE I

	DAYS	1	3	5	7	9	11	13	15	MAXIMUM VARIATION
Case 1		2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	0.0
Case 2		1.9	1.9	1.9	1.8	1.9	1.9	1.9	1.9	0.1
Case 3		2.7	2.7	2.5	2.5	2.7	2.8	2.7	2.7	0.3
Case 4		2.0	2.0	2.0	2.0	1.9	2.0	2.0	2.0	0.1
Case 5		1.9	1.9	1.9	1.8	1.9	1.9	1.7	1.8	0.2
Case 6		2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.4	0.1

Examples of the Behavior of the Index in Disease.—In a case of chronic nephritis (No. 3297, Chart 2) there was a marked nitrogen retention. On admission to the hospital the index was 1.4 at which time the patient was on a low nitrogen diet. He was then placed on a diet consisting solely of fruit juices, and within three days his index rose to 2.8 and promptly fell within twenty-four hours when he was again placed on a low nitrogen diet. During this period there had been no change in either the clinical condition or the blood chemistry. In the second, Case 9477 the clinical diagnosis was chronic

nephritis, hypertension and cardiac decompensation. As is shown in Chart 2 there were variations in the index with a gradual tendency over the period of seventeen days to reach a higher level. During this period, however, there was no decisive change either for better or worse in the patient's condition.

Changes in the index of a much more marked character were also observed in a case of Banti's disease (Chart 3, Case 35433) during an observation period of nineteen days. The significance of these variations could not be determined since the clinical condition of the patient remained practically unchanged.

In a case of hyperthyroidism (Chart 4, Case 35506) rather marked variations of the index were also observed during the fifteen-day period of observation. There was a general tendency of the index to run to successively higher

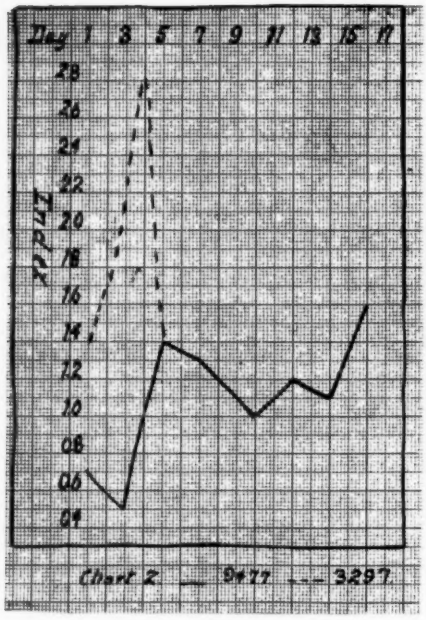

Chart 2

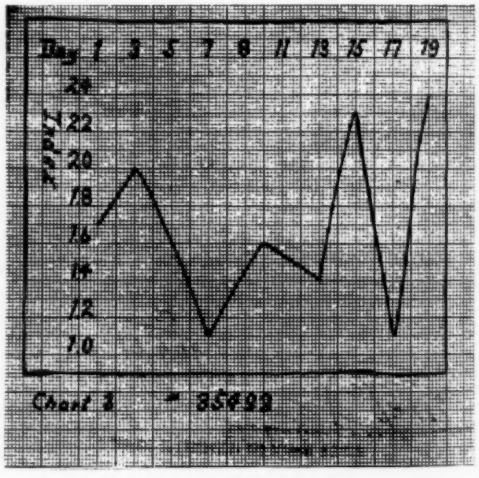

Chart 3

values. This rise in the index was associated with a clinical improvement in the patient.

A further identification of the various factors which may influence the filtration index is afforded in Case 35043, Chart 5. This patient was admitted to the hospital with a diagnosis of chronic cholecystitis with cholelithiasis. The first index taken gave a value of 1.2. Five days later the index was again determined on a specimen taken about two hours after the administration of the dye used to demonstrate the gallbladder roentgenographically, and the index was found to be 3.0. Four days later the index had fallen to 1.5. Four days after the third index was determined the patient was operated upon and the index determined on a specimen taken twelve hours postoperation. The index had fallen to 0.6. The index was again determined three and seven days postoperation and a rise first to 1.8 and then to 2.7 was

demonstrated. The significance and application of the fall in the index directly postoperative and the subsequent rise is discussed in the subsequent paper by Drs. Dujat and Kahan.

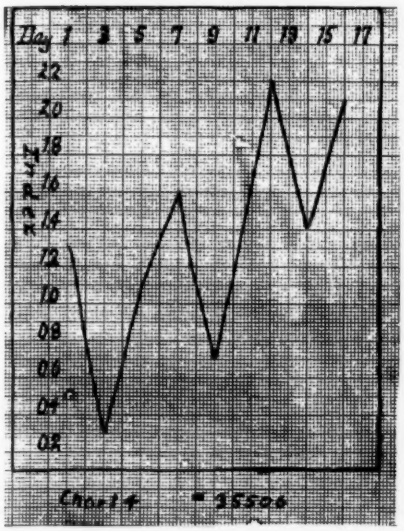

Chart 4

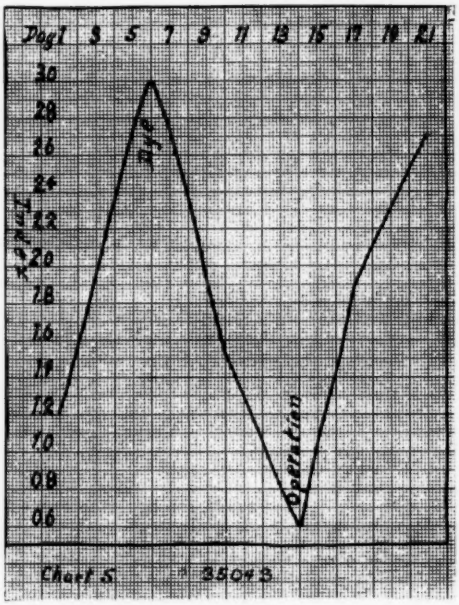

Chart 5

The influence of parenteral injection of foreign protein is shown in Case 35445, Chart 6. This patient was admitted to the hospital with a diagnosis of subacute arthritis of undetermined etiology. Vaccine therapy was given on the dates indicated. The termination of the vaccine therapy was followed by a sharp drop in the index.

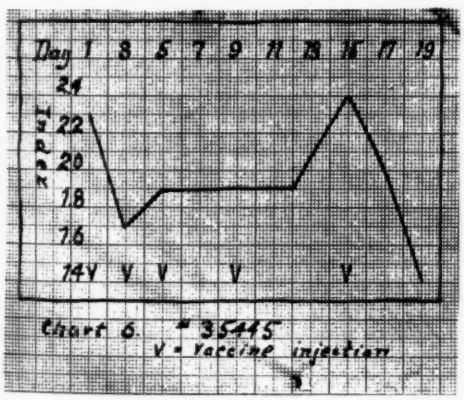

Chart 6

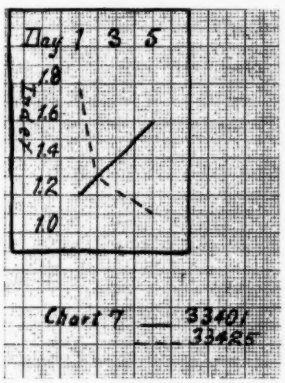

Chart 7

Suggestive of the use of the index in diagnostic differentiation is the curve in the Cases 33401 and 33425. Both of these patients were admitted to the hospital with a history of generalized pains, sore throat and fever.

Case 33401 steadily improved after admission and coincidental with the clinical improvement the index rose from 1.2 to 1.6. The discharge diagnosis was acute follicular tonsillitis. Case 33425 on the other hand did not improve clinically and during the five days of observation the index dropped from 1.8 to 1.1. On the fifth day a diagnosis of scarlet fever was established. The data are graphically presented in Chart 7.

The behavior of the index in a group of cases associated with fever is shown in Charts 8, 9, and 10. Case 33012 (Chart 8) rheumatic fever. The index was determined for the first time on February 26, when it was 1.6. The attack was then rapidly subsiding and the temperature was almost normal. On February 27, the temperature remained normal for twenty-four hours for the first time, and the index dropped to 0.9. On March 1, 3, and 7 the index was respectively 1.4, 1.5, and 1.7. On March 3, the patient was clinically well.

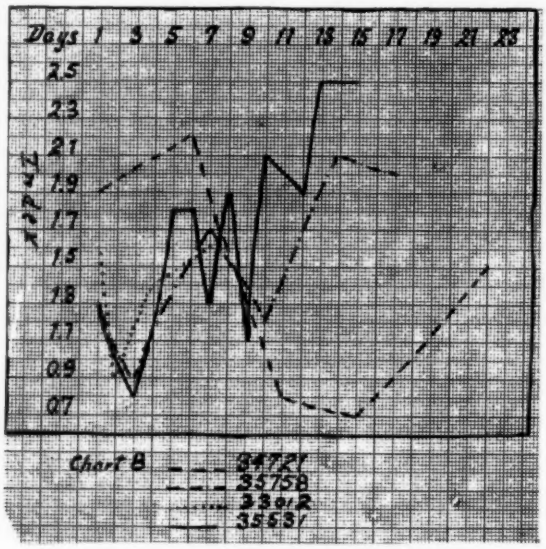

Chart 8

In this case there was a sharp drop in the index the first day of normal temperature. This drop was followed by a progressive rise.

A similar type of reaction was observed in Case 34721 (Chart 8). This patient was admitted to the hospital August 9 with a diagnosis of typhoid fever. When the index was first determined on September 16 the patient was approaching the end of a relapse and the index was 0.8, and four days later it was 0.7. The first rise came on October 3 when it was 1.1 and on October 7 it was 1.5.

Another example of the same type of reaction is shown in Case 35758. This patient was admitted to the hospital November 26 with a diagnosis of thrombophlebitis of the femoral vein. The first index was determined December 3 and was 1.3. The first day of completely normal temperature was on December 5 when the index was 0.9. The indices on December 9, 12, 16, and 19, were respectively 1.7, 1.2, 2.1, and 2.0. The curve is graphically shown in Chart 8.

Still another example is shown in Case 35531 (Chart 8). This patient with lobar pneumonia was admitted to the hospital on October 29. The first index was taken November 3 and was 1.3. The first day of completely normal temperature was November 5 when the index was 0.8. The indices on November 7, 8, 9, 10, 11, 12, 14, 15, 16, and 17 were as follows: 1.8, 1.3, 1.9, 1.1, 2.1, 1.9, 2.5, 2.5, and 2.5.

Still another example is given in Chart 9, Case 34501, one of pneumonia in which the index dropped from 1.3 to 0.7 on the first day of normal temperature. The index does not apparently change markedly previous to a decided change in the patient's condition which occurs suddenly as is shown in Case 33096, Chart 9. This patient had a lobar pneumonia and had an index of 2.0 just a few hours before a fatal cardiac collapse. Another case of lobar pneumonia, Case 35554, Chart 9, shows another phase of reaction of the index.

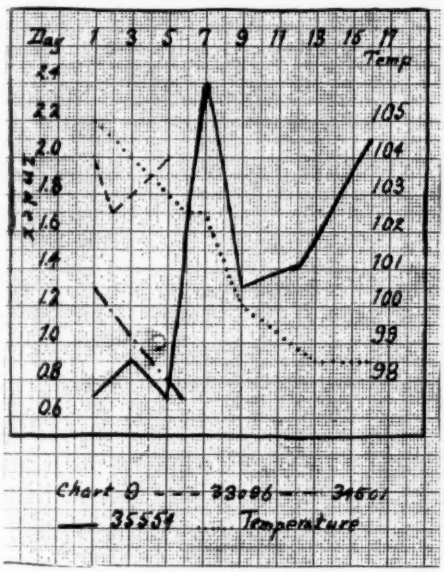

Chart 9

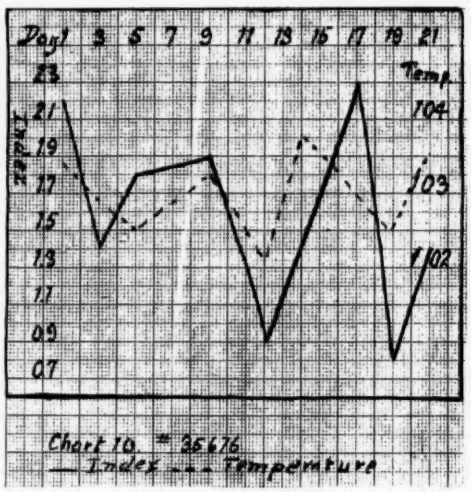

Chart 10

This patient who was very gravely ill had an index varying between 0.7 and 0.9, while the temperature was dropping from 105° to 102.5° F. With the temperature still at 102.5° F., there occurred a rather sudden and very marked improvement in the patient's clinical condition and coincidental with this the index rose to 2.4, to drop forty-eight hours later to 1.3. From this point the index rose steadily to 2.1, the rise antedating the return of the temperature to normal.

In long lasting bacterial infections, in which from the onset it is known that the outlook is unfavorable, as in bacterial endocarditis, fluctuations in the index associated with variations in temperature are also observed. Thus in the patient in Case 35676 (Chart 10) one of bacterial endocarditis, admitted to the hospital November 15 and dying February 4, this was observed. The first index was taken on December 1, and between then and December 21 the index was repeatedly determined. The relation of the temperature curve,

and the curve of the index is graphically shown in Chart 10. In the patient in Case 35428, also bacterial endocarditis, admitted to the hospital October 19 near the termination of the condition, the first index on October 26, was 0.8 while two days later it was 0.5. On October 30, a transfusion was given and while this has in most other instances been followed by a sharp rise in the index, it was in this case followed by a further drop to 0.4. Twenty-four hours after the time of the last index the patient died.

CONCLUSION

If light of differing wave lengths be passed through nonhemolyzed blood serum a definite absorption of the available light occurs. The rate at which the light is absorbed, when calculated for bands 100 Å units in width has been termed the "light filtration index."

In normal individuals this index remains fairly constant over periods as long as fifteen days, minor variations occurring in consequence of simple physiologic disturbances such as constipation, menstruation, etc. The normal index varies between 1.0 and 3.1 with an average of 2.1.

The index is affected by the parenteral injection of foreign proteins, by the ingestion of colloids such as dyes and probably by still other factors.

In pathologic conditions associated with fever the termination of the fever is characterized by a sharp drop in the index and convalescence by a steadily rising index.

An index steadily between 2.2 and 2.6 is of good prognostic import as is a steadily rising index. A consistently low index is of bad prognostic import.

The changes which have been observed in the study of the phenomenon thus far warrant an intensive study in specific disease groups of the behavior of this biophysical change in blood serum so that definite laws guiding in the interpretation of the results may be established.

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CHRONIC MYELOSIS IN CHILDREN*

With Report of a Case

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Baar and Stransky[1] in their well-known Klinische Haematologie des Kindesalters, state that "myelogenous leukemia is well known, but exceedingly rare." In the age table of Reprint No. 143 of the Cancer Commission of Harvard University, the base of the age curve does not touch the 1-9 year line. For this reason we feel justified in reporting an additional case of this disease, and in presenting, in tabulated form, all the cases previously reported in the literature.

CASE HISTORY

The patient was a Jewish boy, nine years of age, admitted to Sydenham Hospital on June 5, 1929, with six months' history of gradually increasing weakness, loss of weight and progressive increase in the size of his abdomen. Aside from slight shortness of breath, induced apparently by his enlarging abdomen, there were no other symptoms. At the beginning of his complaint he was seen by an outside physician who ordered a blood count, with the following findings: Hemoglobin 35 per cent, erythrocytes 2,140,000, leucocytes 425,000, thrombocytes 520,000. The differential smear showed 41 per cent immature neutrophiles, 4 per cent mature neutrophiles, 3 per cent eosinophiles, 3 per cent basophiles, 33 per cent neutrophilic myelocytes, 4 per cent basophilic myelocytes, 10 per cent myeloblasts, 1 per cent monocytes, and 1 per cent lymphocytes.

Six weeks before his admission to this hospital the boy was admitted to Mt. Sinai Hospital, where he stayed two weeks. An abstract received by us of his history there showed a diagnosis of chronic myelogenous leucemia.

The boy's past history showed a normal full-term delivery, weighing 11 pounds, with normal development thereafter. He was breast fed for one year and then put on the regular house diet. His childhood diseases included measles, chickenpox, and whooping cough. The family history was irrelevant.

Physical examination showed a young boy with good physical development and fair nourishment. He was rather pale, giving the appearance of one who is chronically ill. There was a slight dyspnea and orthopnea. He did not cough, and there was no cyanosis. His temperature by rectum was 101.6° F., pulse 116 per minute, and respiration 24 per minute.

The positive findings included marked deviation, to the right of the nasal septum. His eyeballs were large and protruded from their sockets. There were no other eye signs. There was marked pulsation of the vessels on both sides of the neck, and in the suprasternal notch, where a systolic thrill could be palpated. The thyroid gland was not enlarged. The heart appeared to be pushed up somewhat, the apex beat being in the fourth interspace inside the midclavicular line. There was diffuse precordial pulsation and a soft systolic murmur all over the precordium. The lungs were normal.

Examination of the abdomen showed it to be enormously distended, and the superficial veins were enlarged. On palpation an enormously hypertrophied spleen was felt reaching

*From the Department of Pathology, Sydenham Hospital.

down to the symphysis pubis and extending over to the right side. To percussion there was only a small area of resonance, just below the liver.

The lymph nodes in the cervical, axillary, epitrochlear, and inguinal regions were all palpable as small granular masses.

Examination of the eyegrounds showed typical leucemic fundi with blurred, hyperemic discs, and highly dilated, tortuous veins. The arterioles were also dilated but to a lesser degree. There were no hemorrhages or exudative spots.

During the patient's stay at the hospital his temperature varied between 97 and 102° F.

Urinalysis at all times showed a trace of albumin with no other findings. The basal metabolic rate was +10. Blood chemistry was normal. A blood count at that time showed 28 per cent hemoglobin, 1,520,000 erythrocytes, 336,000 leucocytes, and 484,000 thrombocytes. The differential leucocyte count showed 10 per cent mature neutrophiles, 16 per cent immature or band neutrophiles, 20 per cent metamyelocytes, 12 per cent neutrophilic myelocytes, 20 per cent basophilic myelocytes, and 22 per cent eosinophilic myelocytes. Numerous blood

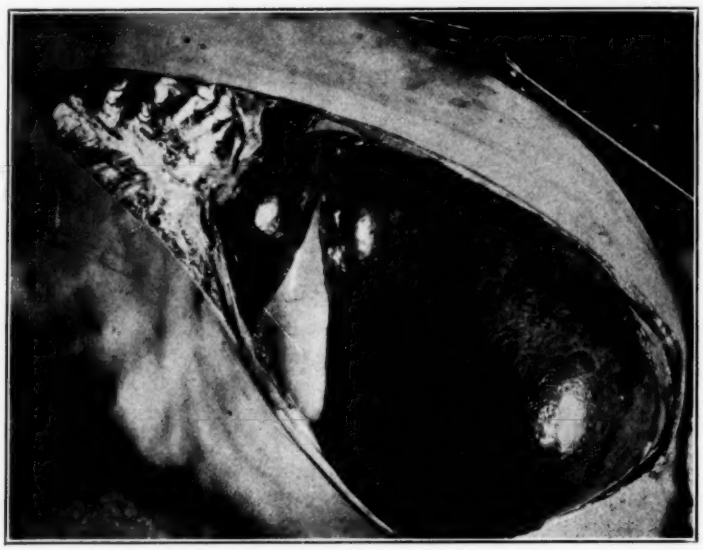

Fig. 1.—Authors' case. Autopsy. Note size of spleen. Weight 5,223 grams.

counts done at different periods during the next two years always showed a similar picture except at one time when the total leucocyte count was brought down to 50,000.

During that time the boy was admitted to the hospital several times and also watched through the dispensary, receiving several courses of benzol treatment and also of deep x-ray therapy to the spleen and long bones.

He received, in all, six transfusions, when warranted by his progressing anemia. They varied in amount from 300 to 500 c.c. and were all given by the Unger method of direct transfusion. The boy was of the Group 1 of the Jansky classification, or the class of universal donors. At each successive transfusion the reaction seemed to be more severe, and when on May 13, 1931, he was given 400 c.c. of carefully cross-matched blood from a professional donor, his temperature rose to 104° F. with a very rapid pulse, chill and hemoglobinuria. He died rather unexpectedly eight hours after the transfusion and approximately two and one-half years after his disease was first diagnosed .

Autopsy performed on April 14, 1931, revealed a large spleen and liver, the former measuring 37 by 21 by 10 cm. and weighing 5,223 gm., while the latter measured 27 by 23 by 8 cm. and weighed 2,680 gm. (Fig. 1). On section of the spleen the cut surface was angry-

red in appearance, with large outstanding granules. Microscopically, there was myeloid metaplasia and infiltration of the spleen, liver, kidneys, lungs, lymph nodes, and bone marrow (Figs. 2 and 3).

General Features of Chronic Myelosis in Children.—Mention has been made of the rarity of this condition. It is interesting to note that, rare as it is, it is exceedingly frequent when compared with chronic lymphadenosis in children. There is but one genuine recorded case of the latter condition. By far the

Fig. 2.—Spleen. Note absence of malpighian corpuscles and normal splenic architecture. Typical myeloid metaplasia present. (Authors' case.)

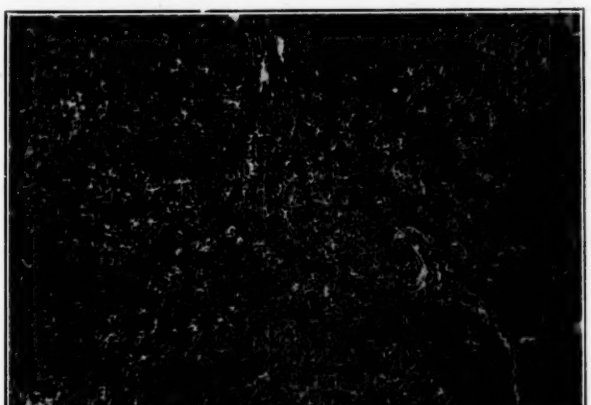

Fig. 3.—Liver. Note sinusoidal infiltration with myeloid cells. This choice of site for infiltration is typical of myelosis as contrasted with the periportal infiltration in lymphadenosis. (Authors' case.)

greatest incidence of leucemia in children lies in the acute types with the acute myelogenous leucemia even more prevalent than the acute lymphatic. The literature includes only 32 cases of chronic leucemia in children. The differentiation, however, between acute and chronic leucemias is often difficult.

It has been repeatedly stressed that in children, even more than in adults, the clinical difference between acute and chronic leucemia, lies not only in the duration of the disease but (and principally so) in the fact emphasized by

Fraenkel[11] that acute leucemias begin with those symptoms with which chronic leucemias end. It must be added, however, that in children chronic myelosis often runs a course so much more rapid than in adults, that on the duration alone one would be tempted to regard it as an acute case, were it not for the absence of the classical symptoms of acute leucemia, namely, hemorrhagic diathesis and an ulcerated mouth. One thing, however, is of great theoretical interest; when a case of chronic myelosis in a child develops the above-mentioned hemorrhagic and mouth symptoms, although the downward progress becomes much more rapid than in a true acute case, the blood picture remains typical of chronic myelosis, even though the case is now an acute one clinically.

Whatever our conception of acute leucemia and its relationship to the chronic form may be, chronic myelosis is a definitely independent clinicopathologic entity. It is a hyperplasia of the myeloid (granulocytic) tissue of the bone marrow and a metaplasia of the myeloid tissue in all the organs where embryonal totipotent mesenchyme is present, myeloid metaplasia in the spleen, liver, and lymph nodes. Hematologically, it expresses itself qualitatively in the presence of immature myeloid cells (granulocytes) from the myeloblast to the metamyelocyte, and quantitatively, in a very high leucocyte count, from 50 to several hundred thousand cells per cubic millimeter. The latter, the very high count, is not an essential feature, since the disease may run its entire course as a subleucemic myelosis with 20 to 50,000 cells, or even as an aleucemic form, with 1 to 10,000 cells per cubic millimeter of blood.

As to the etiology of this extremely interesting condition, though many attractive theories have been proffered, none have been associated with definitely proved facts, and we still know nothing that even begins to approximate certainty. The many hypotheses include the infection theory, as championed by von Hansemann, Manta, Mosler, Fontana and others, the belief that it is a neoplastic process, as claimed by Ribbert,[26] Banti, Warthin, Mallory and Piney, the tuberculosis theory, and lastly the theories of Ziegler[31] and of Naegeli[23] who believe that the disease is a disturbance of tissue correlation and can be traced to disturbed endocrinal relationships.

For a time the most alluring hypothesis was that which regarded all chronic leucemias, the myeloses and lymphadenoses, as a neoplastic process. Piney,[26] however, sounded the keynote of this theory when he said, "it seems the neoplastic hypothesis presents the *fewest difficulties.*" True, from the viewpoint of monophasic anaplasia, leucosis would be regarded as a neoplastic process, but there are too many objections from other points of view, for such a theory to be more than a pretty parallelism. Some of these objections are the absence of infiltrative qualities in the invaded tissues, lack of close relationship to trauma or chronic irritation, the work of Ellerman in transmitting fowl leucoses, remissions and exacerbations, the nature of the hyperplasia, which involves the entire tissue system, over a wide area, rather than an organ, and finally the occurrence of the so-called "leucemoid" blood picture, at times indistinguishable from a true leucemia, yet returning sooner or later to the normal. One also recalls numerous authentic observations of leucemia where the

TABLE I

AUTHOR	CASE	SEX	AGE	CLINICAL PICTURE	BLOOD PICTURE	NECROPSY
Bass[2]	1	F	8	One year history of loss of weight and appetite. Irritating cough. Pale, sallow, undernourished. Superficial nodes large, hard, discrete. Liver and spleen very large.	Hg 41% R.B.C. 2,080,000 W.B.C. 134,000 80% myeloblasts 5 neutrophiles 15 lymphocytes	None
Berghinz[3]	2	F	8	Nine months of small glands, spleen to pubis, large liver, some fever.	R.B.C. 2,000,000 W.B.C. 800,000 27% myelocytes 59 neutrophiles 2 eosinophiles 9 monocytes	None
Cambell[4]	3	M	6	Three months of lymphomegaly. Liver large. Spleen enlarged to navel.	R.B.C. 2,200,000 W.B.C. 1,470,000 36% myelocytes 46 neutrophiles 5 eosinophiles 10 basophiles 2 lymphocytes	None
Cassell[5]	4	F	8	Palpable glands. Liver 2 cm. below costal margin. Spleen to umbilicus. Bones tender.	R.B.C. 3,550,000 W.B.C. 500,000 69% myelocytes	None
Charon and Gratia[6]	5	F	11	Marked enlargement of liver and spleen.	R.B.C. 880,000 W.B.C. 305,000 37% myelocytes	None
Duperie and Cadenaule[8]	6	M	9½	Measles, pneumonia, whooping cough, bronchitis, enteritis bronchopneumonia prior to admission. Headaches, anorexia, abdominal pain, pallor, large liver, and spleen. Treated with x-ray and benzol. Died after four months with hemorrhagic symptoms.	Hg 50% R.B.C. 2,410,000 W.B.C. 264,120 35% myelocytes	None
	7	F	13	Began abruptly with pain in spleen. Loss of weight. Fever. In region of spleen 2 tumors both hard, large and immovable. Splenic puncture showed leucemic picture.	W.B.C. 206,410 reduced by deep therapy to 9,000 4% myelocytes 6 erythroblasts	None
Flesch[9]	8	M	13	Seven months' history of fever with spleen enlarged to pubis.	R.B.C. 2,870,000 W.B.C. 230,000 26% myelocytes	None
Fowler[10]	9	F	5	Five and one-half months of moderate lymphomegaly with enlargement of liver and spleen.	R.B.C. 2,700,000 W.B.C. 240,000 13-30% myelocytes 39-69 lymphocytes	None
Hutchison[12]	10	F	5	Five and one-half months of enlarging abdomen with enormous spleen. No liver and lymph node involvement.	R.B.C. 2,425,000 W.B.C. 1,599,000 69% myelocytes	None

TABLE I—CONT'D

AUTHOR	CASE	SEX	AGE	CLINICAL PICTURE	BLOOD PICTURE	NECROPSY
Japha[14]	11	M	7½	Sick for months. Began with swelling of abdomen and pain. Infantilism. Hard splenic tumor, smooth and tender. Leucemic retinitis.	Hg 35% R.B.C. 3,810,000 W.B.C. 261,000 Smear showed mostly granular cells with later many monocytes.	None
Koch[15]	12	M	10 wk.	Liver two fingerbreadths below the costal margin. Spleen reached to umbilicus.	Hg 28% R.B.C. 2,550,000 W.B.C. 20,900 Platelets 17,000 7% myelocytes 54 lymphocytes	None This is a doubtful case.
Langsch[16]	13	F	10	Pain in left hip, left facial paralysis, ankle clonus, very large splenic tumor. Nerve and joint pains due to local leucemic infiltration. Much improvement under deep therapy.	Hg 59% R.B.C. 3,100,000 W.B.C. 224,000 48% myelocytes	None
	14	M	9	Died with symptoms of hemorrhagic diathesis.	W.B.C. more than 1,000,000	None
Ledingham and Kerron[17]	15	M	11	Four years of enormously enlarged spleen.	R.B.C. 3,570,000 W.B.C. 234,000 48% myelocytes	None
Malmberg[18]	16	F	8 mo.	Father syphilitic. Liver large. Spleen to umbilicus. Septic fever. Hemorrhagic signs before death. Axillary node showed myeloid metaplasia. Duration ten months.	Hg 28% R.B.C. 1,550,000 W.B.C. 177,200	None
	17	M	6 mo.	Pallor at 5 months. Tenderness in head and body. Generalized lymphomegaly. Spleen to iliac crest. Liver enlarged. One and one-half years' duration.	Hg 35% R.B.C. 1,800,000 W.B.C. 356,000 90% myelocytes and myeloblasts	Typical chronic myelosis
Meyer and Heineke[19]	18	M	4	Insidious onset. "Gland" trouble at one year. Four weeks' enlargement of abdomen with occasional nasal hemorrhage. Diffuse adenomegaly. Numerous petechiae. Colossal spleen. Large liver. Death one year later.	Hg 52% R.B.C. 3,080,000 W.B.C. 141,000 Many monocytes, eosinophiles, basophiles, and neutrophilic myelocytes.	Typical chronic myelosis. First case with autopsy.
Middleton[20]	19	M	1¼	Eight months' history of weakness, loss of weight and abdominal swelling. Fever. Constipation and vomiting. Marked anemia. Spleen very large. No glands.	R.B.C. 3,330,000 W.B.C. 1 to every 9 R.B.C.	Typical chronic myelosis

TABLE I—CONT'D

AUTHOR	CASE	SEX	AGE	CLINICAL PICTURE	BLOOD PICTURE	NECROPSY
	20	M	4	Six months' history of abdominal enlargement. No hemorrhages, vomiting or rickets. Spleen reached to umbilicus and to pubis. Heavy, hard with a smooth notch. No nodules.	R.B.C. 3,000,000 W.B.C. 1 to every 5 R.B.C.	Typical chronic myelosis
Morse[21]	21	M	1	Gradual failure of health with enlargement of abdomen and inability to lie on left side. Marked anemia. Adenomegaly. Spleen filled entire abdomen.	R.B.C. 2,900,000 W.B.C. 48,000 22% myelocytes Many erythroblasts	Typical chronic myelosis
Morse[22]	22	M	4	Six months of irregular fever with swelling of joints. Greenish pallor. Spleen to ant. sup. spine. Died suddenly one month after onset of enlarging abdomen.	No blood studies	Typical chronic myelosis
Opitz[24]	23	M	7	Sickly since age of one. Perirectal abscess, otitis media, gradual emaciation. Spleen and liver enlarged. Treated with sunbath and x-ray. At seven was undernourished, body was pigmented, spleen and liver large and hard. Died a year later.	At age of two, 80,000 W.B.C. At age of seven, Hg 70% R.B.C. 3,700,000 W.B.C. 46,000 51% myelocytic cells	None
	24	F	8	At five noticed pallor, listlessness, loss of appetite, ascites and adenomegaly. Liver enlarged. Spleen 13 cm. in diameter.	Hg at one time was 2 per cent (sic!) W.B.C. 37,000 to 460,000 Myeloblasts never over 5%.	None
	25	F	10	Facial paralysis. Left ankle clonus. Very large spleen. Ascites.	W.B.C. 224,000 50% myelocytic cells	None
	26	M	2¾	Exudative skin manifestation. Indistinct speech followed by loss of speech. Large abdomen, cervical and inguinal adenomegaly. Liver to umbilicus. Spleen 2 cm. from midline and down to pelvis. Duration over six months.	W.B.C. 141,000 37% myelocytic cells 10% erythroblast	Typical chronic myelosis
Smith[27]	27	M	13	Four years' duration. Spleen enlarged enormously, reaching into pelvis. Improved under benzol treatment.	W.B.C. 372,000 Many myelocytes and eosinophiles. No myeloblasts. After benzol W.B.C. dropped to 10,000	None

TABLE I—CONT'D

AUTHOR	CASE	SEX	AGE	CLINICAL PICTURE	BLOOD PICTURE	NECROPS
Solmitz[30]	28	M	1	Father a cured (?) syphilitic. Onset with increasing weakness and G-I disturbances. Spleen and liver large. No fever. Died one and one-half years later with hemorrhagic diathesis.	Details not given except many myelocytes. Thrombocytes 35,000 with bleeding time of seventeen minutes	None Malmberg[18] thinks this leucemoid reaction of congenital syphilis.
Steinbrinck and Stukowski[31]	29	M	12	Began with sensation of fullness in stomach after eating. X-ray therapy brought quick and great improvement. Three years later recurred. Died one year later with symptoms of hemorrhagic diathesis.	R.B.C. 2,840,000 W.B.C. 304,000 26% myelocytes 64 neutrophiles	None
van Westrienen[33]	30	F	4	Gradual onset with anemia, large spleen to pelvis, liver moderately large.	Hg 35% R.B.C. 2,720,000 W.B.C. 528,000 54.1% myelocytes	None
Authors' case	31	M	9	Increasing weakness, loss of weight, increasing size of abdomen. Spleen filled entire abdomen. Improved under benzol, deep therapy, and transfusions. Course of two and one-half years with sudden death after sixth transfusion.	Hg 28% R.B.C. 1,520,000 W.B.C. 336,000 Platelets 484,000 74% myelocytic cells	Typical chronic myelosis

blood picture would become normal under the influence of an intercurrent infection, and Jaffe[13] correctly stated that "these facts are difficult to reconcile with the conception that leucemia belongs to the malignant tumors." Finally, the tuberculosis theory is to be mentioned, only to be dismissed as being without any proof.

Despite the lack of positive evidence thereto, we still feel that the most possible progress toward discovering the etiology of this condition, will be made along the work of Ziegler and of Naegeli, on the basis that there is a disturbance in the endocrinal correlationship with abnormality of the internal secreting organs.

COMMENT

At the conclusion of the review of seemingly authentic cases, a few impressions appear justifiable. In the first place the mutual incidental relationship between chronic myelosis and lymphadenosis in children is the same, though markedly accentuated, as in adults, there being only one case of chronic lymphatic leucemia in children as against thirty-three of the myeloid type. Again, acute leucemias (which are much less common in adults than are the chronic forms) are, conversely, more frequent in children. So far as the aleucemic forms of leucemia are concerned, they are much more common, both in children and in adults, in myelosis.

In order of their frequency, therefore, the various forms of leucemia would appear as follows:

CHILDREN	ADULTS
1. Acute myelosis	1. Chronic myelosis
2. Acute lymphadenosis	2. Chronic lymphadenosis
3. Chronic myelosis	3. Acute myelosis
4. Chronic lymphadenosis	4. Acute lymphadenosis

In children, at least, the relationship of incidence according to sexes, is three males to two females.

In going over the clinical picture one becomes impressed with the fact that by far the most frequent complaint in early cases is "weakness, lassitude, listlessness and pallor." In more advanced cases it is "emaciation, enlarged

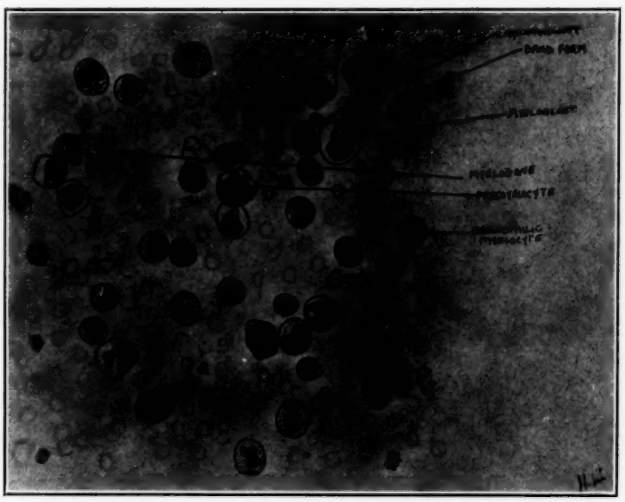

Fig. 4.—Authors' case. Drawing from oil immersion study of blood smear. Actual field.

abdomen" and very frequently "inability to lie on the left side." The spleen is always markedly enlarged, while hepatic enlargement varies from "slight" to "very marked."

A very striking feature of the history in a large majority of cases are numerous infections, from which the children had suffered previously, not infrequently with one infection following immediately upon another (see Cases 8 to 23). In fact a history of several infections succeeding each other at short intervals should always call for a thorough blood examination.

Adenopathy is not a frequent or a prominent symptom, and neither is fever.

The blood picture in chronic myelosis has been described innumerable times, and everyone is acquainted with the high leucocytosis, the immature granulocytes (metamyelocytes, myelocytes, promyelocytes and myeloblasts) and the increase in eosinophiles and basophiles (Fig. 4). In children, there is, perhaps, a greater frequency in the appearance of the youngest or myeloblast forms. This is probably to be accounted for by the great irritability of the hemo-

poietic system in children. The aleucemic form of the disease is quite rare. Anemia is always present and, frequently, is quite severe. It appears from the perusal of the literature that many cases reported as chronic myelosis were in reality cases of von Jaksch-Hayem disease.

Prognosis as to life is, of course, hopeless, and as to duration, variable, some cases lasting as long as five and six years, while others, especially those with a subleucemic count, lasted from a few months to two years.

In treatment, benzol, thorium and deep therapy are variable in their effects on different patients, but as a rule, they always react well to the x-ray treatment, the blood picture promptly changing toward the normal for varying periods of time. At times the anemia is so severe as to require several transfusions (authors' case). In connection with the latter it must be pointed out that toward the end of the disease accidents will occur despite the care with which the grouping and compatibility tests are carried out. Undoubtedly, they are due to some reaction on the part of the leucocytes, since according to Doan[7] there is a definite increase in the fragility of these cells in leucemias.

Just prior to death there almost always appear signs of hemorrhagic diathesis.

SUMMARY

1. There is presented an additional case of chronic myelosis in a child of nine years who responded to treatment for two years, and then succumbed following his sixth transfusion, due in all probability to the increased fragility of the leucocytes.

2. Thirty other authentic cases of chronic myelosis in children are collected from the literature and presented in Table I.

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STUDIES ON THE QUANTITATIVE ESTIMATION OF BILE PIGMENTS*

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IN 1916, Hooper and Whipple[1] developed a quantitative modification of Salkowski's[2] test for bile pigments whereby bilirubin and biliverdin were estimated together. They diluted specimens of bile with a nitro-hydrochloric acid alcohol mixture† and after about 18 hours estimated colorimetrically the characteristic blue green color that had developed on standing. In 1921, Rous and McMaster[3] introduced an inorganic standard‡ to replace the original artificial color wedge§, and later Sribhishaj, Hawkins, and Whipple[4] adopted the use of a monochromatic light filter in reading the intensity of the color in the solutions. Verifying the observations of Whipple and his associates, Riegel, Johnston, and Ravdin[5] in 1932 found that increasing the temperature hastened the reaction. They also found that the "characteristic blue green" color developed long before the maximum intensity was reached.

Further work in this laboratory on the estimation of bile pigment revealed significant inherent errors in current methods, the results of which emphasize the need for this study of the fundamental principles involved in the colorimetry of bile. For example, the time required to develop the color change with the acid alcohol mixture was far too variable to set any definite time at which to make colorimetric readings. One specimen of the acid alcohol may produce the complete color change in less than an hour while another may require more than a week. The color formed in from eighteen to

*Received for publication, June 27, 1933.
This study was made possible by a grant and the facilities of the Research Laboratory of the Children's Fund of Michigan and the Children's Hospital of Michigan.

†Two c.c. conc. HCl, 0.4 c.c. conc. HNO_3, and 100 c.c. 95 per cent ethyl alcohol.

‡Ten c.c. 10 per cent copper sulphate plus 0.075 c.c. of 1 per cent potassium bichromate.

§Made with copper sulphate, india ink, and gelatin agar. On cooling the wedge was sealed with vaseline and standardized against pure bilirubin.

twenty-four hours presents such a wide variety of green shades that frequently colorimetric comparisons are impossible without grossly violating the general laws of colorimetry.[6] At times, the color may be yellowish green, deep green, blue green, or blue. The standard of copper sulphate and potassium bichromate is a constant shade of blue green so that the shade of the unknown rarely matches that of the standard unless perchance by accident. There are also many different shades of blue green, and accurate comparisons are difficult, if not impossible, even when that color is produced. The original standard was prepared by Rous and McMaster[3] with 10 per cent copper sulphate to which they added, immediately preceding the quantitative colorimetric readings, more or less potassium bichromate as the occasion required in order to shift the tint of the standard to correspond with that of the individual unknown. It has been found that their procedure of changing the standard to match the shade of the unknown does materially alter the results. Sribhishaj, Hawkins, and Whipple[4] employing the Wratten Monochromatic Light Filter No. 72[4] found that readings could be made without delay, thus obviating the trouble of adjusting the standard to match each individual pigmentary solution. Their results involved the same error as Rous and McMaster's owing to the type of filter applied. The blue rays only are transmitted with Filter No. 72 which absorbs all other light rays, thereby obliterating the shade of color and permitting the observer to compare the blue intensity of any solution with that of a blue or blue green standard. Since the pigmentary value of the yellow intensity is not recorded, when varying quantities of yellow bichromate are added to the blue copper sulphate standard and when a light filter which records the blue intensity only is used, it seems reasonable to assume that the failure to record the yellow color is a source of an appreciable experimental error.

Although bile is potentially a polychromatic fluid, bilirubin is the parent chromogenic substance, and only those colors which are directly concerned with the oxidation reaction of pure bilirubin are considered in this study. When the pure pigment is dissolved in a simple oxidizing solution, the color change progresses from yellow through green and blue to an ultimately colorless solution. Proper classification of these colors is prerequisite to a better understanding of the colorimetric procedure which follows. The present scientific classification of all color is based on the discovery of the solar spectrum[7] in 1666 by Sir Isaac Newton, who found that sunlight was composed of many colors, which could be separated when the sun's rays were allowed to pass through a glass prism. Working with Newton's discovery, Sir David Brewster[8] in 1831 showed that the visible spectrum contained only three primary colors, namely, red, yellow, and blue. By means of light filters, which he had developed in 1822,[9] he demonstrated that the orange, green, and violet colors of the spectrum were secondary, produced by the overlapping of red and yellow, yellow and blue, and blue and red rays, respectively. The fundamental principle that green is a secondary color composed of two primary colors, yellow and blue, was utilized in the present study in the preparation of standards for the colorimetric estimation of all

green shades developed by the oxidation of bilirubin. This principle is applied by the quantitative separation of the yellow and blue intensities from a composite green solution by means of monochromatic light filters.*

Experimental Procedure.—A series of dichromatic green standards were prepared by combining definite quantities of yellow and blue dye solution.† The yellow dye was standardized against a known amount of pure unoxidized bilirubin‡ and the blue against the maximum blue intensity developed from an equal quantity of oxidized bilirubin. These solutions were then mixed so that each successive standard contained the equivalent of a 10 per cent increase in blue and a compensating 10 per cent decrease in yellow. The resulting ratio of yellow to blue produced therefore a different shade of green in each member of the series. The Wratten Filter No. 76 was used for standardizing and reading the yellow in the solutions and No. 72 was used for the blue.

With these standards, an attempt was first made to ascertain the causes of the difficulties encountered with Hooper and Whipple's acid alcohol mixture, and second to devise an accurate method for determining bile pigment. A quantitative study of the color change in all phases of the reaction was developed along two major lines, namely, the factors which influence the reaction velocity and those which control the variation in shades. With the aid of light filters, experiments on the time required to complete the color changes were made with various oxidizing solutions as well as with different types of nitric acid as the oxidizing agent. Observations were also made upon the effect of temperature and light. The reaction velocity was determined by the amount of blue developed during any given period of time and the end

*The Wratten Monochromatic Filter No. 72, when placed over the eyepiece of the colorimeter allows the observer to record only the intensity of the blue rays. With the Wratten Monomatic Filter No. 76, one records only the intensity of the yellow rays.

These filters were obtained from the Eastman Kodak Co., Rochester, N. Y.

†*Preparation of Standards.*—

I. Stock solutions:

1. Pure bilirubin, 25 mg. in 100 c.c. of chloroform.
2. Dimethylamidoazobenzene, 32 mg. in 100 c.c. ethyl alcohol.
3. Nile blue sulphate, 7.5 mg. in 100 c.c. ethyl alcohol.
4. Nitrohydrochloric acid alcohol[1] made with strictly fresh nitric acid.

II. Standardization of diluted stock solutions:

1. Dilute 20 c.c. of the stock yellow dye to 500 c.c. with alcohol.
2. Dilute 20 c.c. of the stock blue dye to 500 c.c. with alcohol.
3. Expose the nitrohydrochloric acid alcohol solution to ultraviolet light for three minutes at 18 inches from the quartz mercury vapor tube. Stir the solution every fifteen seconds. The depth of the solution not to exceed 5 cm.
4. From a microburet, measure 1 c.c. of the stock bilirubin solution into each of ten 25 c.c. volumetric flasks.
5. Dilute 5 flasks to the mark with plain ethyl alcohol.
6. Dilute the other 5 flasks to the mark with the treated nitrohydrochloric acid alcohol solution. Allow to stand one hour, at which time they should all be steel blue in color.
7. With the first 5 flasks of unoxidized bilirubin solutions as standards, determine the bilirubin equivalent of the yellow dye. If the average for the series does not closely approximate the known amount of bilirubin, adjust the intensity and restandardize. Use light filter No. 76.
8. With the second 5 flasks of oxidized bilirubin, determine the bilirubin equivalent of the blue dye. Restandardize if necessary. Use light filter No. 72.
9. From a buret measure accurately and mix the yellow and blue dye solutions as follows:

Standard No.	0	1	2	3	4	5	6	7	8	9	10
C.C. Yellow	100	90	80	70	60	50	40	30	20	10	0
C.C. Blue	0	10	20	30	40	50	60	70	80	90	100

10. Label each standard according to its value in terms of oxidized and unoxidized bilirubin.

‡Obtained from the Eastman Kodak Company, Rochester, N. Y.

TABLE I

A COMPARATIVE STUDY OF THE QUANTITATIVE ESTIMATION OF BILIRUBIN AS INFLUENCED BY HNO_3

Method 1: Estimations made with single copper sulphate and potassium bichromate standard with the aid of filter No. 72. Solution contains 1.040 mg. per cent bilirubin.

Method 2: Estimations made with a series of dichromatic green standards with the primary colors mixed in an inverse ratio as described in the text.

SOLUTION NUMBER	OXIDIZING SOLUTION*	TEMPERATURE	LIGHT	REACTION TIME	COLOR	ESTIMATIONS IN MILLIGRAMS PER CENT	PER CENT ERROR	STANDARD NUMBER	YELLOW INTENSITY (BILIRUBIN) FILTER NO. 76	BLUE INTENSITY (BILICYANIN) FILTER NO. 72	BILIRUBIN PLUS BILICYANIN	PER CENT OF OXIDATION†	PER CENT ERROR‡	STAGE OF OXIDATION
1	HNO_3 1 yr old	Room	Lab. Daylight	15 min.	Blue Green	1.099	+ 5.67	9	0.068	0.990	1.058	95.1	+1.75	Late 2nd
2	HNO_3 Fresh	Room	Lab. Daylight	24 hr.	Yellowish Green	0.320	−69.23	3	0.692	0.341	1.033	32.7	−0.67	Middle 1st
3	HNO_3 Fresh	Room	Dark Room	24 hr.	Yellowish Green	0.321	−69.13	3	0.682	0.343	1.025	32.9	−1.43	Middle 1st
3	HNO_3 Fresh	Room	Dark Room	48 hr.	Green	0.404	−61.15	4	0.585	0.452	1.037	43.4	−0.28	Late 1st
3	HNO_3 Fresh	Room	Dark Room	72 hr.	Green	0.667	−35.86	5	0.533	0.490	1.023	47.1	−1.63	Early 2nd
3	HNO_3 Fresh	Room	Dark Room	7 days	Blue Green	0.715	−31.25	7	0.276	0.753	1.020	72.4	−1.92	Middle 2nd
4	HNO_3 Fresh	Room	Lab. Daylight	7 days	Blue Green	0.811	−22.01	8	0.225	0.827	1.052	79.5	+1.09	Middle 2nd
5	Same lot as No. 3	38° C.	Dark Room	24 hr.	Green	0.622	−40.19	6	0.379	0.672	1.051	64.6	+1.05	Early 2nd
5	Same Lot as No. 3	38° C.	Dark Room	48 hr.	Blue Green	0.901	−13.36	8	0.170	0.883	1.053	84.8	+1.25	Middle 2nd

*95 per cent ethyl alcohol 100 c.c.; HCL 2 c.c.; HNO_3 0.4 c.c.
†Calculated from blue intensity.
‡Calculated from both yellow and blue.

TABLE I—CONT'D

SOLUTION NUMBER	OXIDIZING SOLUTION*	TEMPERATURE	LIGHT	REACTION TIME	COLOR	ESTIMATIONS IN MILLIGRAMS PER CENT	PER CENT ERROR	STANDARD NUMBER	YELLOW INTENSITY (BILIRUBIN) FILTER NO. 76	BLUE INTENSITY (BILICYANIN) FILTER NO. 72	BILIRUBIN PLUS BILICYANIN	PER CENT OF OXIDATION†	PER CENT ERROR‡	STAGE OF OXIDATION
5	Same Lot as No. 3	38° C.	Dark Room	72 hr.	Blue Green	1.029	− 1.05	9	0.102	0.968	1.070	93.0	+2.88	Late 2nd
5	Same Lot as No. 3	38° C.	Dark Room	7 days	Blue	1.050	+ 0.09	10	—	1.029	1.029	98.9	−1.05	Early 3rd
6	Same Lot as No. 3 and 5	38° C.	Dark Room	24 hr.	Green	0.559	−46.25	6	0.409	0.614	1.023	59.0	−1.63	Early 2nd
6	Same Lot as No. 3 and 5	38° C.	Dark Room	48 hr.	Blue Green	0.763	−26.63	7	0.256	0.790	1.046	75.9	+0.57	Middle 2nd
6	Same Lot as No. 3 and 5	38° C.	Dark Room	72 hr.	Blue Green	0.878	−15.57	8	0.170	0.889	1.059	85.4	+1.82	Middle 2nd
6	Same Lot as No. 3 and 5	38° C.	Dark Room	7 days	Blue Green	0.938	− 9.81	9	0.085	0.995	1.080	95.6	+3.84	Late 2nd
7	HNO_3 Fresh Ultraviolet 1 min.	Room	Lab. Daylight	2 hr.	Green	0.617	−40.67	5	0.502	0.546	1.048	52.5	+0.76	Early 2nd
7	HNO_3 Fresh Ultraviolet 1 min.	Room	Lab. Daylight	18 hr.	Blue	1.105	+10.09	10	—	1.046	1.046	100.5	+0.57	3rd
8	HNO_3 Fresh Ultraviolet 2 min.	Room	Lab. Daylight	2 hr.	Blue Green	0.996	− 4.23	8	0.162	0.885	1.047	85.0	+0.70	Middle 2nd
8	HNO_3 Fresh Ultraviolet 2 min.	Room	Lab. Daylight	4 hr.	Blue	1.094	+ 4.23	10	—	1.046	1.052	101.1	+1.15	3rd

of the color change was determined by that point at which the maximum blue intensity was reached. The green color in all phases of the oxidation reaction was recorded in terms of both yellow and blue which permitted a study of the relationship of shade to the ratio existing between the two primary colors. It was then possible to determine the percentage of oxidized bilirubin both by the increase in the blue intensity and by a decrease in the yellow. A few typical experiments are reported.

Experimental Data.—The characteristic color change which takes place when pure bilirubin is dissolved in a simple oxidizing solution is due to its combination with oxygen from either the solution or the surrounding atmosphere. The speed with which the reaction takes place depends upon the oxidizing agent and a definite relationship exists between the reaction velocity and the availability of the oxygen. The oxidizing solutions which furnish this element in a readily available form bring about the color change without delay, while the more stable reagents which release the oxygen slowly or with difficulty produce the color transformation at a correspondingly retarded rate.

The observations on nitric acid were especially significant since it has been the reagent of choice in the quantitative estimation of bile pigment. Although it is a powerful oxidizing agent, the oxygen may not always be in a readily available form, a fact which explains the extremely wide variations found in the oxidation reaction time with bilirubin. The amount of oxygen which is free to unite with the pigment depends upon the decomposition of the acid which liberates variable quantities of nascent oxygen and nitrogen dioxide on long-standing, with heat or in sunlight. Consequently, these three factors control the reaction velocity. A specimen of nitric acid which was partially decomposed by standing in the laboratory for about a year, when made up in the usual acid alcohol mixture developed the "characteristic blue green" color in fifteen minutes. This is shown by the first solution in Table I which had actually undergone 95.1 per cent oxidation in that time. In contrast, strictly fresh nitric acid always required more than a week to complete the oxidation reaction as shown by Solutions 3 and 4, which were only 72.4 and 79.5 per cent, respectively, oxidized in seven days. Different specimens of ordinary nitric acid produced various results in the reaction time, depending upon the degree of decomposition of the acid, but always ranging within the extreme limits of fifteen minutes on the one side to a week on the other. Some specimens produced the "characteristic blue green" color in from twelve to eighteen hours as reported by Hooper and Whipple,[1] while others required from eighteen to twenty-four hours as given by Rous and McMaster.[3] Most specimens, however, brought about the color alteration at an entirely different velocity, *characteristic of the particular type of nitric acid.*

By comparing the typical results recorded for Solutions 2 and 3, it may be noted that ordinary daylight in the laboratory had no effect in hastening the color change, whereas heat increased the reaction by facilitating the decomposition of the nitric acid as shown by Solutions 3, 5, and 6. Solutions 5 and 6 incubated at 38° C. were 64.6 and 59.0 per cent, respectively, oxidized in

twenty-four hours, whereas Solution 3, as a control at room temperature, was only 32.9 per cent oxidized in the same length of time. This gives an increase in the reaction velocity of 96 and 79 per cent respectively due to increased temperature. Certain specimens of nitric acid produced the complete oxidation of the bilirubin in four hours at 38° C. as reported by Riegel, Johnston, and Ravdin.[5] At that temperature, however, strictly fresh nitric acid always required more than three days to complete the color change, thus emphasizing that heat is only one of the three determinative factors in bringing about the color transformation by the liberation of oxygen from nitric acid.

Irradiation of the acid alcohol mixture with ultraviolet light produced the same changes in its reaction time with bilirubin as did sunlight, heat, or long-standing. Preliminary observations showed that the nitric acid was the only constituent in the acid alcohol mixture that was influenced by this treatment. Bilirubin, however, was rapidy decolorized by irradiation, making it necessary to observe every precaution in preventing the light rays from coming in contact with the pigment, and therefore treatment of the acid alcohol mixture was carried out in another room. With ultraviolet light, it was possibe to regulate at any desired speed the oxidation reaction velocity of fresh nitric acid with bilirubin. Irradiation of the acid alcohol for ten minutes brought about the complete color change in four minutes, but at this velocity a lowering of the maximum blue intensity occurred from loss of pigment by rapid oxidation. The time of exposure was gradually decreased until it was found that treatment of the acid alcohol for three minutes produced the maximum blue intensity in forty-five minutes with no appreciable loss of pigment, while the same solution without irradiation required more than seven days. Light treatment for two minutes developed the complete color change in four hours and for one minute in eighteen hours, showing a direct relationship between the length of time the acid alcohol mixture was exposed to ultraviolet light and the reaction velocity of the oxidation of bilirubin.

Comparison of the curves in Chart 1, which records the development of the blue intensity by the oxidation of bilirubin with nitric acid after one, two, and three-minute exposure of the acid alcohol mixture to ultraviolet light, showed that the contour was essentially the same as curves recording the effects of heat or long-standing on the same solution. The time required to complete the oxidation reaction was the only variation, a fact which is illustrated by superimposing the control curve, plotted in hours instead of minutes, over a curve having a more rapid velocity accelerated by irradiation (Chart 1). In all instances, the oxidation reaction velocity was comparatively rapid early in the process, becoming slower as the supply of available oxygen diminished. Calculations made from the data recorded for Solution 3, Table I, show that the strictly fresh nitric acid used in this solution accomplished 45.5 per cent of the total oxidation during the first twenty-four hours, and 14.4 per cent in the second twenty-four hours with an average of 8 per cent per day for the remainder of the week. When the nitric acid was sufficiently decomposed with ultraviolet light, as shown in Curve 3, Chart 1, the supply

of oxygen set free was sufficient to bring about the complete color change without delay, whereas when the oxygen was released slowly by the more stable specimens of nitric acid, complete oxidation of the bilirubin was greatly prolonged. Insufficient decomposition of the acid by irradiation, formed the blue intensity early with increased speed, carrying the first portion of the curve relatively high, depending upon the amount of liberated oxygen, which as it became exhausted, checked the velocity of the reaction, causing the blue intensity to rise much more slowly later, as shown by Curves 1 and 2. Regardless of the degree of decomposition of the nitric acid, the trend of the curve was always progressively upward until the point of maximum blue intensity was reached.

These observations on the development of the blue intensity are of special interest in relation to the various shades of green formed. For purposes of description one may divide the complete oxidation reaction of bilirubin into four arbitrary phases, based upon the value of the blue in-

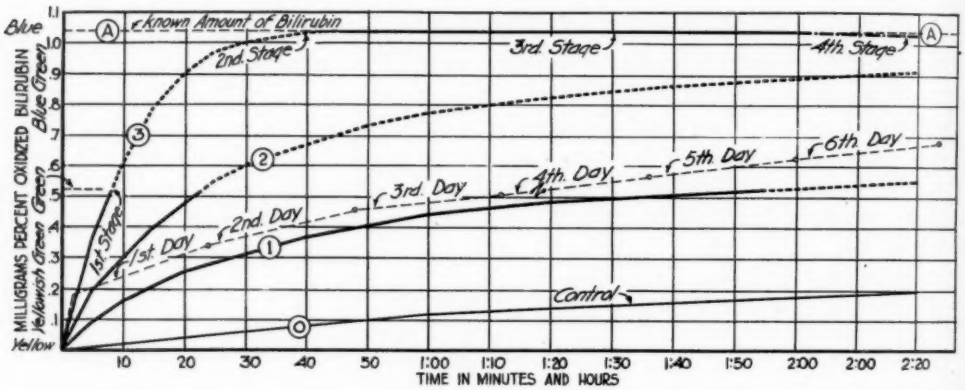

Chart. 1.—Illustrating in Curves 1, 2 and 3 the progressive development of the blue color in the oxidation of bilirubin with nitric acid alcohol mixtures which have been exposed to ultraviolet light for one, two, and three minutes, respectively. Each curve is divided into different stages: The first stage, which is represented by the heavy solid line, is the yellowish green phase, during which the yellow intensity predominates over the blue; the second stage, which is represented by the heavy broken line, is the blue green phase, during which the blue intensity predominates over the yellow; the third stage is the steel blue, during which the blue color remains at its maximum; the fourth stage, which is represented by the heavy dot and dash line is the phase during which the blue gradually fades out. The light solid line, *O*, shows the oxidation of bilirubin by the control or untreated acid alcohol mixture; the light dash and circle curve is marked off in hours instead of minutes and represents the slow oxidation of the control solution over a period of six days.

tensity as an index to the extent of the oxidation: the first, comprising all the various shades of yellowish green, begins with the oxidation of the first molecule of bilirubin and ends when 50 per cent of the pigment has been oxidized; the second, consisting of all the various shades of blue green, begins as a continuation of the first stage and ends at the point of maximum blue intensity, indicating complete oxidation of the bilirubin; the third represents that portion of the oxidation reaction during which the blue intensity remains maximum; the fourth, as shown at the end of Curve 3, illustrates the gradual fading out of the blue color (Chart 1). The *color change* as defined in this paper comprises the first two stages, since the third is constant and the fourth stage is characterized by a fading out of the blue color. The "characteristic

blue green'' color described by Hooper and Whipple[1] appears early in the second stage, long before the maximum blue intensity is reached, an observation also made by Riegel, Johnston and Ravdin.[5] Since the former investigators used the intensity of the blue color during this stage as a base for their calculations in the quantitative estimation of bile pigment, one should note that consecutive quantitative estimations are not constant when made upon the blue intensity of the same solution during the blue green stage. This observation is also illustrated by Solution 8, Table I, which grossly was definitely well advanced in the blue green stage long before the initial two-hour reading was made, but showed an increase of 15.8 per cent in the blue intensity during the second two-hour period. From the foregoing observations one may conclude that the *blue green color represents a stage of incomplete oxidation of the bilirubin, and quantitative estimations calculated upon the basis of the blue intensity only, do not represent the total pigment content because the unoxidized bilirubin remains unrecorded.*

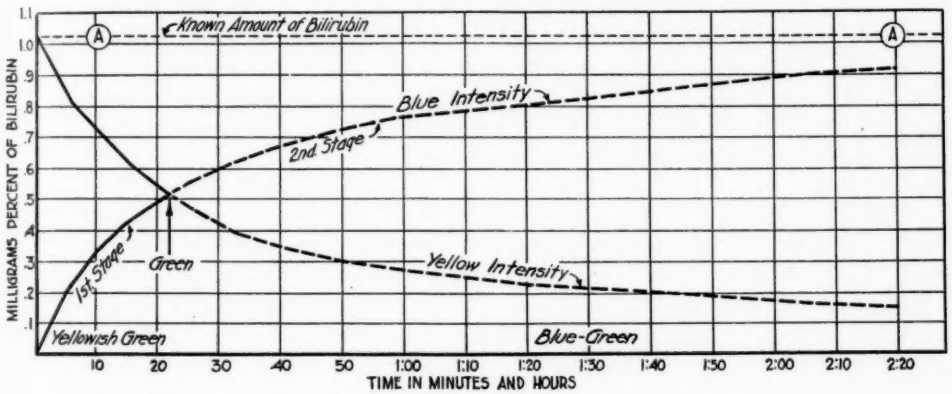

Chart 2.—Illustrating the quantitative relationship between the yellow and blue intensities in the same green solution during the color change produced by the progressive oxidation of pure bilirubin with nitrohydrochloric acid alcohol mixture. The heavy solid line shows the first stage, during which the yellow is greater than the blue and the various shades of yellow green appear. The rapid fall in the yellow was quantitatively compensated for by an equally rapid rise in the blue, so that the two curves met when exactly one-half of the bilirubin was oxidized. The heavy dashed line which represents the second stage in the oxidation of bilirubin begins at the point where the two curves cross or at the point where the blue begins to progressively predominate over the yellow and forms the various shades of blue green.

In order to understand more fully the significance of the various shades of green formed by the oxidative process, studies on the yellow intensity were a necessary counterpart to the studies on the blue. Companion quantitative studies were therefore made of both the yellow and the blue intensities on the same solution, as shown in Chart 2, which illustrates the typical incomplete reaction of nitric acid with bilirubin. The curve representing the quantitative value of the yellow intensity is practically an inverted mirror image of the blue and barring the slight unavoidable experimental errors in quantification could be inverted and superimposed upon the line representing its counterpart. The rapid fall in the yellow intensity was quantitatively compensated for by an equally rapid rise in the blue, so that these two curves crossed when exactly half of the bilirubin had been oxidized, indicating that yellow and blue are the only primary colors involved in the oxidation reaction.

This observation agrees with the statement made earlier in this paper that green is a secondary color composed of two primary colors, yellow and blue. The solution was a perfect deep green color at the point at which the curve for the yellow intensity crossed that of the blue, for at this point the values of the two intensities were colorimetrically equivalent. Up to this point, which marks the end of the first stage, the yellow intensity quantitatively predominated over the blue and the solution developed a variety of yellowish green shades subject to the extent of the oxidation. Following the point of pigment equilibrium which was marked by the perfect green color, the blue became more and more dominant over the yellow as the oxidation progressed, developing an apparently even greater variety of blue green shades because of the slower reaction velocity during the second stage. Due to rapid oxidation early in the reaction, the first stage lasted only twenty-two minutes, whereas the second occupied several hours, finally reaching the stage of maximum blue intensity when the last traces of yellow had disappeared from the solution.

The relation of the predominant primary color to the recessive one depends upon the extent of the oxidation and control the exact shade of green developed throughout the oxidation reaction of bilirubin. By a study of the data presented in Table II, one finds this relationship to be an inverse ratio, which explains how gentle oxidation through a gradual fall in the yellow intensity and a compensating rise in the blue insidiously changes one shade of green to another. Theoretically, the shade of green is never twice the same during the continuous reaction. One can readily see that unless the reaction velocity of each solution were exactly the same, the shades would be different. This explains the difficulties encountered when matching green bile pigment solutions against one another in the colorimeter. It also explains why a series of green standards with the primary colors mixed in an inverse ratio are essential for accurate colorimetric estimation of all the possible shades of green that may be developed.

By quantitatively estimating the components of the green color separately and combining the results as recorded in Table II, one finds that the estimated value of the yellow intensity plus the blue corresponds closely to the known amount of pigment in the initial solution, a finding of practical importance in the quantitative estimation of bile pigment. With this method, regardless of the shade of color or the extent to which the bilirubin is oxidized, providing it has not yet passed the stage of maximum blue intensity, the pigmentary value of the solution can be accurately estimated at any time during the oxidation reaction. The experimental error, however, was increased slightly at the beginning and at the end of the color change, probably due to the physical difficulty of reading small amounts of yellow in a predominantly blue solution and vice versa, whereas no difficulty was encountered where the yellow to blue ratio was more equalized.

The importance of considering the yellow intensity in the quantitative estimation of bile pigment is reflected in Table I by a comparison of the experimental error recorded after estimating solutions with a known pigment content, both by the older method and by those used in this investigation.

TABLE II

PROGRESSIVE OXIDATION OF A 1.024 MILLIGRAMS PER CENT SOLUTION OF PURE BILIRUBIN WITH THE NITROHYDROCHLORIC ACID ALCOHOL MIXTURE AS THE OXIDIZING SOLUTION

TIME 1:10 P.M. 1/26/33	VISIBLE COLOR	STAGE OF OXIDATION	STAND. NO.	YELLOW INTENSITY		BLUE INTENSITY		BILIRUBIN PLUS BILICYANIN	PER CENT OF OXIDATION	PER CENT ERROR
				ESTIMATED UNOXIDIZED BILIRUBIN CORRECTED FOR 1 MINUTE*		TIME P.M.	ESTIMATED OXIDIZED BILIRUBIN (BILICYANIN)			
1:16	Yellowish green	Early 1st	2	0.811	0.035*	1:15	0.201	1.047	19.6	2.27
1:21	Yellowish green	Middle 1st	3	0.712	0.019	1:20	0.333	1.064	32.5	3.97
1:26	Yellowish green	Middle 1st	4	0.604	0.021	1:25	0.424	1.049	41.4	2.48
1:31	Green	Late 1st	5	0.530	0.015	1:30	0.488	1.033	47.6	0.87
1:36	Green	Onset 2nd	5	0.465	0.013	1:35	0.547	1.025	53.4	0.09
1:43	Bluish green	Early 2nd	6	0.384	0.010	1:42	0.619	1.023	60.4	0.09
1:50	Bluish green	Early 2nd	6	0.354	0.008	1:49	0.664	1.023	64.8	0.19
2:00	Bluish green	Middle 2nd	7	0.304	0.009	1:59	0.721	1.034	70.4	0.97
2:10	Blue green	Middle 2nd	7	0.268	0.005	2:09	0.763	1.036	74.5	1.19
2:20	Blue green	Middle 2nd	8	0.254	0.001	2:19	0.778	1.033	75.9	0.87
2:30	Blue green	Middle 2nd	8	0.226	0.002	2:29	0.803	1.031	78.4	0.68
2:40	Blue green	Middle 2nd	8	0.219	0.0007	2:39	0.821	1.0407	80.1	1.64
2:52	Blue green	Middle 2nd	8	0.203	0.001	2:51	0.852	1.056	83.1	3.11
3:01	Blue green	Middle 2nd	9	0.180		3:00	0.871	1.051	85.0	2.68
3:16	Blue green	Late 2nd	9	0.161		3:15	0.902	1.063	88.0	3.80
3:30	Blue green	Late 2nd	9	0.148		3:29	0.920	1.068	89.8	4.38

*Calculated as the average decrease per minute for the preceding five minutes.

For example, when Solution 3 was estimated in twenty-four hours, by the copper sulphate and potassium bichromate standard with Filter No. 72, which records the intensity of the blue color only, the estimated pigmentary value was found to be 0.320 mg. per cent, giving an error of 69.23 per cent. In contrast, when compared in the colorimeter with standard No. 3, the same solution was found to be 32.7 per cent oxidized with a yellow and blue concentration of 0.692 mg. and 0.341 mg. per cent respectively, giving a total pigment content of 1.033 mg. per cent, with an experimental error of 0.67 per cent. This table illustrates that the yellow intensity in the green solutions which corresponds to the unoxidized bilirubin fraction if ignored is recorded as a part of the experimental error.

The observations on the green color formed by the oxidation of bilirubin made during this investigation differ somewhat from the usual impressions given in the textbooks. It is generally implied that biliverdin is the direct oxidation product of bilirubin. These experiments do not entirely contradict this, but tend to explain the manner in which the color of biliverdin is actually formed. The total oxidation of bilirubin completely transformed the yellow solution to a blue color commonly known as bilicyanin, while the partial oxidation formed a great variety of green shades, depending upon the extent of the oxidation. Based upon the present scientific classification of colors, these studies indicate that biliverdin is a mixture of bilirubin and bilicyanin rather than a definite entity. Coordinate quantitative studies on both the yellow and the blue intensities in the same solution illustrate that the oxidation reaction which was characterized by a gradual and progressive shifting in color from yellow through green to blue was accompanied by a quantitative transformation of bilirubin to bilicyanin.

SUMMARY

Stimulated by difficulties encountered in the colorimetric methods for the quantitative estimation of bile pigments, a study of the quantitative color change through all phases of the oxidation of bilirubin has been made. The factors influencing the reaction velocity of the oxidation of bilirubin, and the factors controlling variations in the resultant shades of green, yellow green and blue green have been investigated by the preparation and use of a series of suitable standards which are composed of definite mixtures of stable blue and yellow dyes.

It was found that the time required to complete the color change from yellow to deep blue varied with the specimens of nitric acid employed which in turn differed in their supply of free oxygen. External factors such as heat, light, and ultraviolet irradiation influenced the rate of oxidation of bilirubin by virtue of their direct effect upon the liberation of free oxygen from the nitric acid. The blue green color generally employed in colorimetric determinations represents a stage in the incomplete oxidation of bilirubin, and hence determinations based upon this method do not quantitatively represent the amount of bile pigment present in the solution. However, by the quantitative estimation of both the blue and the yellow components, with the aid of light filters and suitable dichromatic standards, the total amount of pigment

in the original solution can be accurately determined at any stage in the oxidation reaction. These experiments indicate that the bile pigment generally designated as biliverdin is a mixture of bilirubin and its oxidation product bilicyanin.

The authors take great pleasure in expressing their sincere appreciation to Icie G. Macy, Ph.D., for her generous aid during the course of this work.

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DEMONSTRATION OF PENETRATION OF AN ANTISEPTIC DYE INTO THE TISSUES OF THE GENITOURINARY TRACT*

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IN VIEW of the fact that the effective results of a urinary antiseptic may be conditioned not only by concentration in which it is bacteriostatic and bactericidal, but also by the ability of the compound to penetrate into the tissues, it was considered to be of some interest to demonstrate such penetration into the tissues lining the genitourinary tract.

Experiments to show such penetration were carried out as follows: Irrigations of urethra and bladder were made on a series of male rabbits, using a 0.5 per cent solution of the dye. This was supplemented by injections into the pelvis of the kidney via ureter before removal of the entire genitourinary tract. The dye used for these experiments was phenyl-azo-diamino-pyridine hydrochloride (pyridium).

In the preparation of histologic sections, difficulty was encountered in preventing the dye from being leached out of the tissues by the various agents used for fixation, hardening, imbedding, and clearing. After numerous experiments with different procedures, it was found that the most satisfactory method was one in which the tissues were fixed in mercuric chloride solutions

*From the Department of Biological Chemistry, Long Island College of Medicine.
Received for publication, May 10, 1933.

and then frozen sections cut, mounted, and immediately photographed. Although other metallic salts also are capable of precipitating the dye, all were found to be more or less unsatisfactory due to the fact that subsequent manipulations of the section leached out the dye from the tissues. Similar objec-

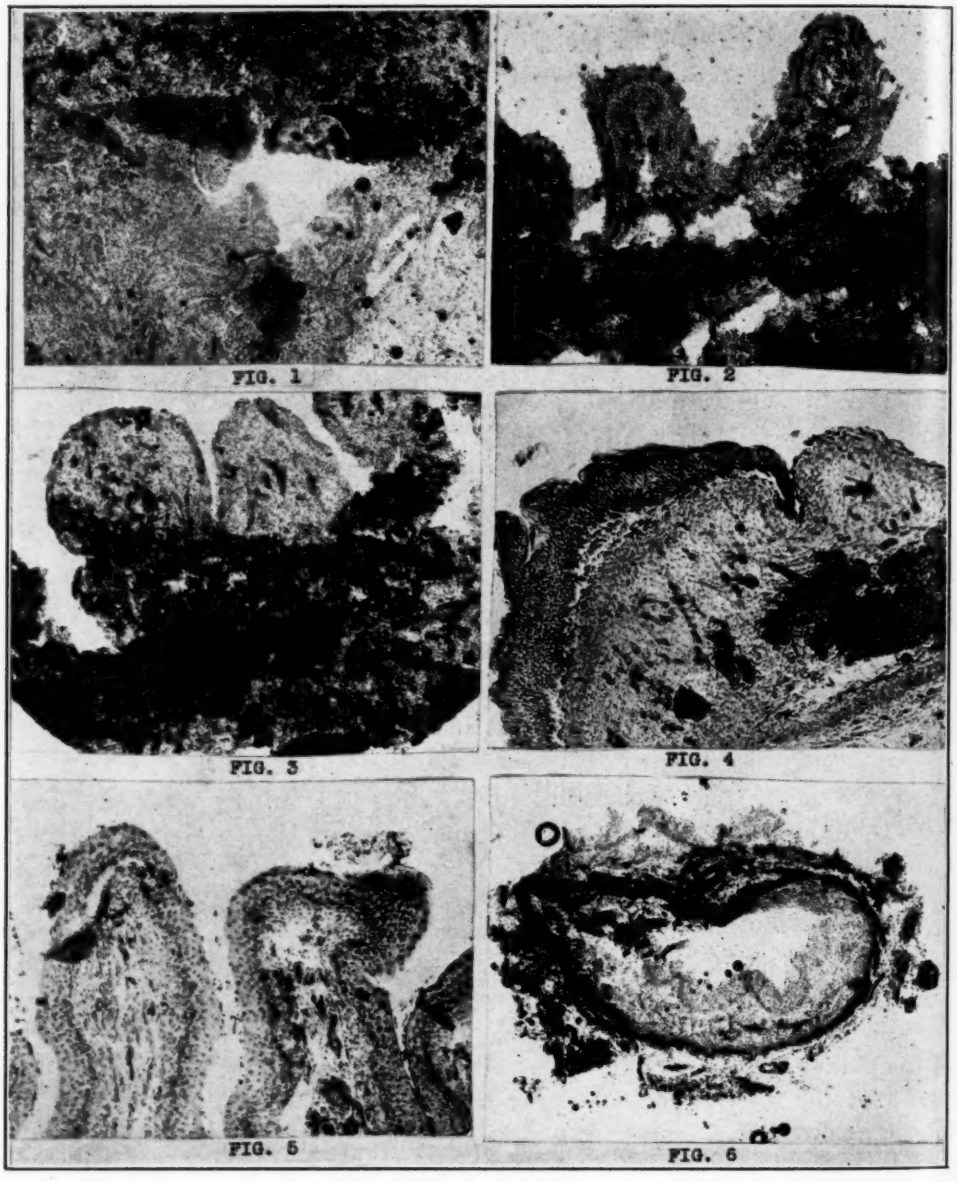

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6

tions were encountered in the case of the alkaloidal reagents, such as picric acid and Lugol's solution, which precipitate the dye, or in the case of alkalies which precipitate the free base.

Mercuric chloride was finally selected since it produced the least soluble, as well as the most highly colored precipitate even in fairly dilute solutions. Before making this selection various other standard fixatives were tried, but

difficulty was always encountered either because the dye was not precipitated or was in fact leached out of the tissues by the fixing solution. Although mercuric chloride was found to be the best precipitant and fixative ordinary imbedding processes involving the use of alcohol and alcohol-ether mixtures for purposes of dehydration were found to bring about solution and diffusion of the mercuric compound of the dye into other nonstained parts of the tissues. The most suitable method for demonstration of the true extent of penetration was found to be as follows: Small portions of excised tissue are placed in bichloride solution for twenty-four hours, frozen sections are then cut, the sections floated in bichloride solution, mounted in glycerin, and photographed.

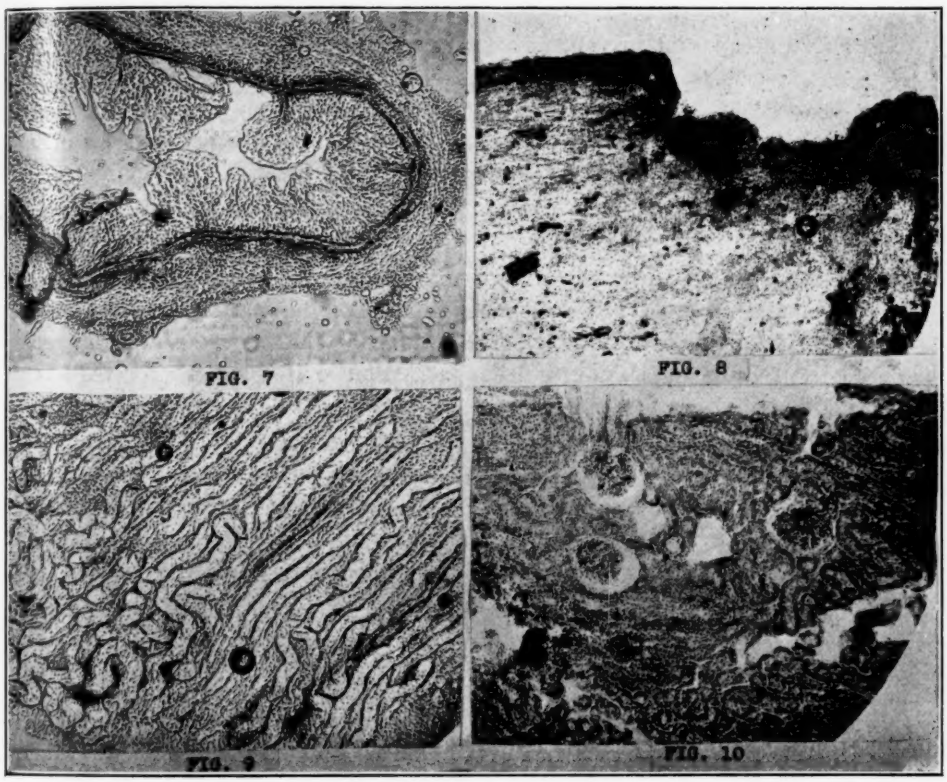

Fig. 1 is a cross-section of the urethra under low power magnification and shows the mercuric precipitate of the dye. Figs. 2 and 3 are sections of bladder wall under low power magnification showing precipitated dye in the tissues. Fig. 4 is also a cross-section of the bladder wall under high power magnification and may be compared with Fig. 5 which is a similar section of normal bladder wall. Fig. 6 is a cross-section of the ureter showing precipitate of the dye and should be compared with Fig. 7, a section of normal ureter. Fig. 8 is a section of kidney tissue adjacent to the pelvis of this organ and shows dye precipitate, whereas Figs. 9 and 10 are sections of deeper portion of the kidney and show no penetration.

SOME EXPERIMENTAL STUDIES ON NEMBUTAL (PENTO-BARBITAL-SODIUM)*

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Charleston, S. C.

IN TESTING a constant pressure apparatus, with which we wished to be able to inject glucose solutions intravenously at a definite rate over long periods of time, it was found convenient to employ an anesthetic. Nembutal was selected, because of its rapidity of action, and shorter acting time than the other barbiturates. Urine was to be collected and tested for reducing substances. It was observed that very little urine was obtainable during the period of narcosis.

Fee[1] has shown that water diuresis established in decerebrate dogs, by the administration of water through the stomach tube, is checked by the administration of chloroform, ether, chloralose, or morphine in doses commonly employed to produce anesthesia, and the inhibition lasts, roughly, for the same length of time as the narcotic effect.

Frey,[2] in his experiments on rabbits, using urethane, chloral hydrate, and morphine, found that diuresis was decreased whether the water was administered by mouth, by rectum, or through an intestinal fistula.

Ogden[3] demonstrated the inhibition of water diuresis by amytal, and Marx[4] concluded that amytal had a strong antidiuretic effect on dogs, and moreover may cause a retention of substances in the blood ordinarily excreted through the kidneys.

Swanson and Shonle,[5] in their pharmacologic study of nembutal (pento-barbital-sodium), pointed out the fact that nembutal is an isomer of amytal, the difference being in the alkyl side chain, the methyl group in the amytal molecule being attached to the γ-c-atom of the side chain, while in the iso-amytal molecule the methyl group is linked to the α-c-atom. The position of this methyl group seemingly has a great influence on the physiologic activity.

The disputed question as to the effect of amytal on blood sugar[6, 7, 8, 9, 10] raised the question as to the effect of pento-barbital-sodium on the blood sugar level of a fasting animal.

Hines, et al.,[11] showed that a greater hyperglycemia and a marked increase of glycosuria existed when glucose solution was injected intravenously under amytal anesthesia than in the control experiments on the same animal; whereas Brill[12] concluded that sodium amytal had a marked inhibiting effect on the hyperglycemia produced by morphine.

*From the Department of Physiology, Medical College of the State of South Carolina.

EXPERIMENTAL

Five mongrel dogs in normal condition, so far as could be determined by blood and urinary laboratory methods and physical examination, were used.

Each dog received 1 c.c. (1 c.c. = 1 gr.) nembutal (pento-barbital-sodium, Abbott) per 5 pounds body weight. The dogs varied from 14 to 27 pounds in weight. The dogs were fed their usual kennel rations at 2:00 P.M. and received no feeding other than water, which was always accessible. They were accustomed to the laboratory and any excitement was avoided as much as possible.

The next morning, about sixteen or eighteen hours after last food given, they were brought into the laboratory, and 3 c.c. of blood was obtained by venous puncture of the saphenous vein, and oxalated to prevent clotting. Serum was obtained by allowing 2 c.c. of blood to clot.

Water ranging from 200 c.c. to 300 c.c., depending on the size of the animal, was given by stomach tube. The bladder was emptied immediately, and emptied at twenty-minute intervals thereafter, and the amount measured and recorded. Normals for urinary output under water diuresis, blood sugar, urea, nonprotein nitrogen, and total serum protein were obtained for each dog.

The blood samples were secured at approximately hourly intervals, and until such time as the animals indicated that they were reacting well from the anesthesia as manifested by attempts to raise head, stand, etc.

The nembutal was injected intravenously at such a rate that the animals were unconscious at the termination of the injection. During the injection, the respiration always quickened. Injecting too rapidly produced a rapid, jerky respiration. The respiration became slow, full, and deep as soon as the animals were unconscious. There never was any period of excitement, and the same results were always obtained on subsequent injections on the same animal.

On the dosage given, 1 c.c. per 5 pounds (1 c.c. = 1 gr.), a surgical anesthesia was obtained in about five minutes and persisted from forty-five to fifty minutes. The dogs remained asleep a variable length of time, but all were showing definite signs of awakening within three and a half hours and usually were on their feet in four to four and one-half hours with only slight ataxia and incoordination persisting.

The normal urinary output, and the output under nembutal anesthesia, have been charted (Chart 1) for each dog to show the decrease in the urinary output.

Only one record for each dog is shown to avoid confusion on the chart, but several records on each were made, and all responded in a like manner. Increasing the dosage of nembutal, as little as 0.5 c.c. resulted in a greater decrease in the urinary output. In Dog 17, increasing the dosage from 3 c.c. to 3.5 c.c. resulted in anuria during the period of narcosis.

With an increase in the total dosage of nembutal a longer period of anesthesia resulted, both in the period of surgical anesthesia and in the time in which reaction was first noted.

In only one instance (Dog 10) did an active diuresis seem to begin after the animal began to react. It is possible that during the longer time in which

narcosis is present sufficient water is lost by other routes, other than through the kidney, so that there is not sufficient excess water present to produce an active diuresis.

Marx, H.[13] states that amytal has an influence on the cerebral centers, regulating metabolism of water, and that it also influences the endothelium of the capillaries, changing the permeability; from which he concluded that the hypnotic directs the water to the tissues of the body in general, instead of

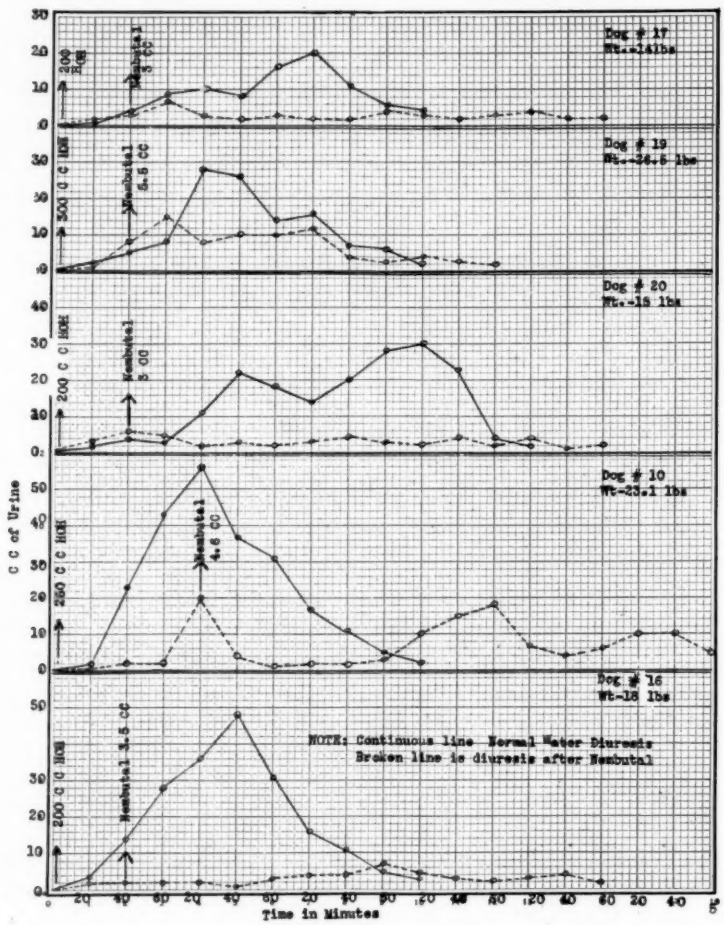

Chart 1

to the kidneys. We are inclined to believe that nembutal has a similar action. This action is not peculiar to the barbiturates alone, as it is possible to produce edema by injecting sodium chloride infusions quite readily, if chloroform, ether, or chloral hydrate has been used to anesthetize the animal.

From Pratt's[14] work, one can infer that the liver breaks down and detoxifies pento-barbital, because in experimental lesions of the liver, produced by chloroform, nembutal in the usual dosage produces prolonged narcosis.

We thought is might be interesting to note the blood chemistry changes, if any, during the period of narcosis, and if repeated injections would result in damage to the liver and kidneys.

Since the blood chemistry studies were in agreement with each dog tested, the results of the study of one dog only are tabulated (Table I).

TABLE I

TIME	DOG 10 BLOOD SAMPLES	SUGAR—NORMAL	SUGAR DURING NEMBUTAL ANESTHESIA	UREA—NORMAL	UREA DURING NEMBUTAL ANESTHESIA	T.S.P.—NORMAL	T.S.P. DURING NEMBUTAL ANESTHESIA	N.P.N.—NORMAL	N.P.N. DURING NEMBUTAL ANESTHESIA
10:00 A.M.	First sample		78.7		20.4		5.2		23.6
10:20	Second sample	64.5	103.2	19.1	22.6	5.4	4.8	25.2	20.8
11:20	Third sample	64.7	76.2	20.6	21.6	5.7	4.53	26.2	18.5
12:20 P.M.	Fourth sample	74.3	88.5	20.3	21.4	5.9	4.41	26.4	23.2
1:20	Fifth sample	76.2	90.5	20.3	21.8	5.7	4.71	26.1	24.2

There was in each instance a slight rise in the blood sugar levels, though not sufficient in our opinion to warrant the conclusion that nembutal raises the blood sugar level. The rises noted might be accounted for by the metabolic changes occurring during that length of time.

Because of the interest and controversy in regard to the effect of amytal on the blood sugar level, the results of the five dogs studied will be tabulated below (Table II).

The blood samples were obtained as follows:

Sample 1, before giving water
Sample 2, 20 minutes after giving nembutal
Sample 3, 1 hour after second sample
Sample 4, 1 hour after third sample
Sample 5, 1 hour after fourth sample

TABLE II

BLOOD SUGAR DURING NEMBUTAL ANESTHESIA

	DOG 16	DOG 10	DOG 20	DOG 19	DOG 17
First sample	55.5	78.7	57.8	85.1	58.3
Second sample	58.2	103.2	66.2	93.4	68.9
Third sample	71.8	76.2	83.4	94.3	64.7
Fourth sample	74.1	88.5	83.5	71.2	64.1
Fifth sample	66.2	90.5	75.6	71.5	63.5

In one of our experiments, in which we were injecting a glucose solution, 6 per cent, intravenously, we found the blood sugar level to rise to 256 mg. per 100 c.c., without producing any diuresis. While further experimentation on this point is necessary, it indicates that the inhibition of water diuresis, produced by nembutal, cannot be broken by infusions of glucose at such a rate that the renal threshold is exceeded.

Three of our dogs have received repeated injections (1 c.c. per 5 pounds [1 c.c. = 1 gr.]) at intervals of six days. Dog 16 has had 8 injections and dogs 10 and 19 ten such injections. The animals did not lose weight and their blood chemistries and urinalyses were within normal limits one week after the last injection.

TO SUMMARIZE

1. Nembutal seems to be an efficient and rapidly producing anesthetic, with no initial period of excitement, with a shorter period of narcosis, and a relatively shorter reacting time than the other barbiturates.

2. Water diuresis in dogs is inhibited by doses of nembutal (pentobarbital-sodium, Abbott), sufficient to produce anesthesia. (Dose: 1 gr. per 5 pounds body weight.) In one of our dogs, increasing the dosage, a complete suppression of urine followed, during the period of narcosis.

3. There is a slight change in the blood sugar during the period of narcosis.

4. Blood chemistry studies, including urea, nonprotein nitrogen, and total serum protein, were not appreciably affected during the period of narcosis.

5. Three dogs, receiving as many as 8 to 10 injections at intervals of six days, showed no changes in hepatic or renal functions that could be detected by the usual blood and urine laboratory examinations.

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THE USE OF HYDROGEN PEROXIDE IN THE QUANTITATIVE ESTIMATION OF BILIRUBIN IN BILE*

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IN A CRITICAL study of the quantitative estimation of bile pigment,[1] it was shown that there are errors in the current colorimetric method[2-5] which are due to the inefficient and variable oxidizing agent employed and the failure to consider the unoxidized pigment fraction which forms a part of the blue green color used in making the colorimetric readings. It was demonstrated that the amount of free oxygen which controls the color change depends upon the degree of decomposition of the different specimens of nitric acid used in making up the oxidizing solution and this in turn produced extremely wide variations in its oxidation reaction time with bilirubin. Consequently, the various degrees of spontaneous decomposition existing in the nitric acid standing in the different laboratories, presents an inconstant variable which renders the results on the quantitative estimation of bilirubin incomparable. Furthermore, it was shown that the changes in the color of the pigment solution were produced by a gradual and progressive oxidation of the bilirubin to bilicyanin and that the green solution commonly known as biliverdin should be considered colorimetrically as a mixture of the two pigments, bilirubin and its oxidation product, bilicyanin, each of which must be estimated separately in order to ascertain the pigmentary value of the green solutions produced in the oxidation reaction.

In the present report an attempt has been made to eliminate the errors due to the variability of the nitric acid by replacing it with hydrogen peroxide which gives a more constant and rapidly available source of free oxygen. The difficulties as well as the errors involved in estimating the pigmentary value of the blue green color are eliminated when the readings are made later in the oxidation reaction, namely, during the stage of maximum blue intensity. The final colorimetric readings with hydrogen peroxide as the source of available oxygen in the oxidizing solution can be made within thirty minutes instead of the former from eighteen to twenty-four hours, thus giving a simplified, rapid and accurate method for the quantitative estimation of the bilirubin in bile.

PREPARATION OF SOLUTIONS

Stock Bilirubin Solution.—Transfer 25 mg. of pure bilirubin† to a clean dry 100 c.c. volumetric flask and make up to volume with chloroform.

Nile Blue Sulphate Solution.—Transfer 15 mg. of nile blue sulphate‡ to a 100 c.c. volumetric flask and make up to volume with 95 per cent ethyl alcohol.

*Received for publication, June 27, 1933.

This study was made possible by a grant and the facilities of the Research Laboratory of the Children's Fund of Michigan and the Children's Hospital of Michigan, Detroit.

†The bilirubin was obtained from the Eastman Kodak Company, Rochester, New York.

‡Nile blue sulphate (diethyldiaminonaphthophenoxazine) $(C_{20}\ H_{20}\ N_3\ O)_2\ SO_4$ made by The Coleman and Bell Company, Norwood, Ohio, was used.

Oxidizing Reagent.—A solution of 2 c.c. of concentrated hydrochloric acid and 0.4 c.c. of 30 per cent hydrogen peroxide* is made up to 100 c.c. with 95 per cent ethyl alcohol.

STANDARDIZATION OF THE DYE SOLUTION

Transfer 1 c.c. of the stock bilirubin solution into each of five 25 c.c. volumetric flasks and dilute to volume with the oxidizing reagent. After thoroughly shaking allow the solution to stand for thirty minutes at room temperature until the blue color develops. Dilute 10 c.c. of the nile blue sulphate solution to 500 c.c. with 95 per cent ethyl alcohol. Using the 1 mg. per cent solutions of oxidized bilirubin as standards determine the bilirubin equivalent of the diluted blue dye solution. The average of the five determinations should be taken as the final result. Place the Wratten Monochromatic Light Filter No. 72† over the eyepiece of the colorimeter when making the readings. Calculate the values for the dye solution by the formula as follows:

$$\frac{\text{Reading of standard} \times \text{Concentration of standard in mg. per 100 c.c.}}{\text{Reading of unknown}} = \text{Bilirubin equivalent of the blue dye in mg. per 100 c.c.}$$

Method for the Determination of Bilirubin in Bile ‡—Transfer 1 c.c. of the bile to be analyzed to a clean dry 50 c.c. volumetric flask and make up to volume with the oxidizing reagent. After thoroughly shaking allow the solution to stand for thirty minutes at room temperature until the blue color develops. Clear the solution of precipitated mucus in the centrifuge or by filtering and compare intensity of the cleared solution in the colorimeter with that of the stable blue standard. Place the Wratten Monochromatic Light Filter No. 72 over the eyepiece of the colorimeter before making the readings. The calculations are made as follows:

$$\frac{\text{Reading of standard} \times \text{Concentration of standard in mg. per 100 c.c.} \times \text{dilution}}{\text{Reading of unknown}} = \text{Mg. of bilirubin per 100 c.c.}$$

The experimental error of the method with known solutions of pure bilirubin is less than 2 per cent. The variation in results with bile itself, however, is increased at times to nearly 5 per cent due to the inability to deliver aliquot specimens of mucus laden fluid from the ordinary pipette. For this reason triplicate determinations should be averaged in order to reduce the experimental error to a minimum and every precaution should be exercised in uniformly draining the pipette.

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*Merck's hydrogen peroxide, 30 per cent (Superoxol).

†Wratten Monochromatic Light Filter No. 72 was obtained from the Eastman Kodak Company, Rochester, New York. The shade of the final blue color developed from fresh bile for the readings is sufficiently constant to eliminate the use of this filter but frequently the final tint is changed by the presence of other pigments in the gallbladder and this filter eliminates such interferences. Filter No. 72 permits the intensity of the blue rays only.

‡Experiments show that the prinicpies of this method may be applied to the quantitative estimation of bilirubin in serum and urine. Further work is in progress on the details of the technic.

DISCUSSION

Although bilirubin is the parent chromogenic substance in bile, there may be other pigmentary substances formed on standing through its oxidation, reduction, hydrolysis, or conjugation with other bile constituents. Due to the fact that bilirubin is an unstable pigment, quantitative estimations should be made upon strictly fresh bile before any spontaneous chemical changes occur to cause the loss of pigment. Because of the natural affinity of bilirubin for oxygen, which it quickly absorbs from the solution or the surrounding atmosphere, spontaneous oxidation is frequently the earliest change that occurs in fresh bile and may even take place within the gallbladder, while it invariably occurs subsequent to removal from the body and exposure to the air. Colorimetric studies indicate that bilicyanin, which is the direct oxidation product of bilirubin, is present in greater or lesser amounts in the specimens of bile which have already turned green from spontaneous oxidation. Although both

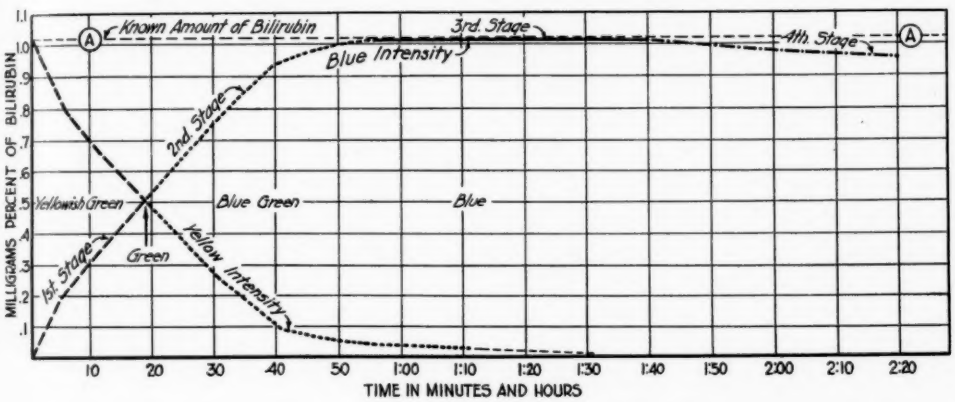

Chart 1.—Illustrating the four stages in the progressive oxidation reaction of a 1.024 mg. per cent solution of pure bilirubin with the hydrogen peroxide-hydrochloric acid-alcohol oxidizing reagent and the relationship of the two primary colors which form the green solution.

The first stage is yellowish green color due to the predominance of the unoxidized bilirubin over its oxidation product, bilicyanin; the second stage is blue green color due to the bilicyanin predominating over the bilirubin; the third stage represents the maximum blue intensity developed from the bilirubin by oxidation and illustrates the most reliable position in the oxidation reaction for quantitatively estimating the total pigment content by means of a single monochromatic blue standard; during the fourth stage the blue intensity gradually fades out.

spontaneous and induced oxidation transform the bilirubin to bilicyanin, it so happens that the oxidizing reacts with both pigments so that the bilicyanin previously formed by spontaneous oxidation may be further oxidized to colorless end-products by the time the remainder of the bilirubin is oxidized to bilicyanin. Experience shows that 2 or 3 per cent of bilicyanin mixed with the bilirubin gives the solution a definite greenish tint, and yet, this amount is within the limits of the experimental error, so that even though the bile specimen is slightly greenish the simple method described above is sufficiently accurate for practical purposes. In specimens which have undergone greater spontaneous oxidation, however, it becomes necessary to make separate colorimetric estimations of the two constituent pigments, bilirubin and bilicyanin, as described in a previous publication[1] and illustrated in Table I.

TABLE I

PROGRESSIVE OXIDATION OF A 1.024 MG. PER CENT SOLUTION OF PURE BILIRUBIN WITH HYDROGEN PEROXIDE HYDROCHLORIC ACID ALCOHOL

TIME 4:00 P.M. 1/12/33	VISIBLE COLOR	STAGE OF OXIDATION	STANDARD NO.	YELLOW INTENSITY ESTIMATED UNOXIDIZED BILIRUBIN CORRECTED FOR 1 MIN.*			BLUE INTENSITY TIME P.M.	ESTIMATED BILIRUBIN (BILICYANIN)	BILIRUBIN PLUS BILICYANIN	PER CENT OF OXIDATION	PER CENT ERROR
4:06	Yellowish green	Early first	2	0.788	0.039*	0.827	4:05	0.190	1.017	18.5	0.68
4:11	Yellowish green	Middle first	3	0.672	0.023	0.695	4:10	0.319	1.014	31.1	0.97
4:16	Green	Late first	4	0.571	0.020	0.591	4:15	0.421	1.012	41.1	1.17
4:21	Green	Onset second	5	0.461	0.022	0.483	4:20	0.539	1.022	52.6	0.19
4:26	Bluish green	Early second	6	0.359	0.020	0.379	4:25	0.642	1.021	62.7	0.29
4:31	Bluish green	Middle second	7	0.254	0.021	0.275	4:30	0.754	1.029	73.6	0.48
4:36	Blue green	Middle second	8	0.169	0.016	0.185	4:35	0.852	1.037	83.2	1.27
4:41	Blue green	Middle second	9	0.091	0.015	0.106	4:40	0.941	1.047	91.8	2.27
4:46	Blue green	Late second	9	0.074	0.003	0.077	4:45	0.979	1.056	95.6	3.11
4:51	Blue green	Late second	9	0.046	0.005	0.051	4:50	1.007	1.058	97.7	3.32
4:56	Blue green	Late second	9	0.041	0.001	0.042	4:55	1.018	1.060	99.4	3.51
5:01	Blue green	Late second	9	0.039		0.039	5:00	1.018	1.057	99.4	3.27
5:06	Blue green	Late second	9	0.036		0.036	5:05	1.018	1.054	99.4	2.92
	Blue	Third	10				5:10	1.018	1.018	99.4	0.58
	Blue	Third	10				5:15	1.018	1.018	99.4	0.58
	Blue	Third	10				5:40	1.017	1.017	99.3	0.68
	Blue	Fourth	10				6:00	0.979	0.979		4.49

*Calculated as the average decrease per minute for the preceding five minutes.

There are four stages in the complete oxidation of pure bilirubin as shown in Chart 1 and Table I. During the first stage, the yellow unoxidized bilirubin predominates over its blue oxidation product, bilicyanin, while during the second stage their ratio is reversed and forms the blue green color generally used in colorimetric comparisons. It has been demonstrated that the blue green color represents a stage of incomplete oxidation of bilirubin, and quantitative estimations made during this stage not only require the determination of the oxidized bilirubin but also the pigment fraction that remains unoxidized.[1]

For such a determination it is necessary to employ the more complicated series of dichromatic green standards which contain the primary colors mixed in an inverse ratio.[1] In contrast, the present accurate yet simplified method for determining bilirubin utilizes the maximum blue intensity of the third stage of oxidation for the colorimetric readings and requires but a single monochromatic blue standard. As the oxidation progresses from the second into the third stage the color changes from the blue green to blue, and the curve for the blue intensity ceases to rise, remaining at the maximum level for considerable time, due to the fact that at this time the available oxygen is nearly depleted and the oxidation velocity is comparatively slow. Since the time limits of the third stage are sufficiently long to permit readings of a series of solutions without undue haste and the blue color remains constant during this stage, it offers, therefore, the most reliable period during the oxidation reaction of bilirubin in which to quantitatively estimate the total pigment content by monochromatic methods.

Through the introduction of hydrogen peroxide to replace the nitric acid in the oxidizing solution, the practicability of using the third stage for the final readings is made possible. Because of its labile oxygen, hydrogen peroxide has the ability to complete the oxidation of bilirubin to bilicyanin within thirty minutes instead of the former eighteen to twenty-four hours. The maximum blue intensity developed with hydrogen peroxide from a known amount of pure bilirubin is remarkably constant, varying only with the amount of pigment in the initial solution. Slight alterations in the amount of hydrogen peroxide do not alter the height of the maximum blue intensity but only serve to increase or decrease the length of time the blue intensity remains at its maximum. The same is true for variations in the hydrochloric acid in the oxidizing solution. In order to make accurate readings on both the yellow and blue colors in the same solution as shown in Chart 1, the reaction time of bilirubin with hydrogen peroxide was prolonged from twenty minutes to fifty-five minutes by reducing the amount of hydrochloric acid in the oxidizing solution from the usual 2 per cent to 1 per cent. With the use of a single monochromatic standard, however, the maximum blue color only is considered, and therefore, the more rapid reaction obtained with higher acid content is the more desirable.

Pure bilirubin may be used as a standard by treating the stock bilirubin solution in the same way as the unknown, but when it is used the solution in the colorimeter cup must be changed with each unknown, as the prolonged catalytic effect of the strong colorimeter light upon the oxidation of the standard

is sufficient to alter the results. Because of this difficulty the procedure is greatly facilitated by using a stable blue dye standard which is not affected by the light. Although the artificial standard is photostabile the oxidation of the pigment in the bile solution is hastened by the influence of strong light, and for this reason the reading of the unknown should be made promptly, once the solution is placed in the colorimeter. A standard composed of an alcoholic solution of an organic blue dye, nile blue sulphate, was found to be preferable to an aqueous solution of the inorganic salt, copper sulphate, which was previously used for this purpose.

SUMMARY

A simplified, rapid, and accurate method for the quantitative estimation of bilirubin in bile is presented in which hydrogen peroxide with its more constant and available source of free oxygen has been substituted for the nitric acid in the oxidizing reagent, thus eliminating errors due to the variability of the different specimens of nitric acid. The final colorimetric readings can be made within thirty minutes instead of the usual eighteen to twenty-four hours and at the maximum blue intensity instead of during the variable blue green color stage, making it possible to use a stable monochromatic organic dye, nile blue, as a standard for quantitatively estimating bile pigment.

The authors take great pleasure in expressing their sincere appreciation to Icie G. Macy, Ph.D., for her generous aid during the course of this work.

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A METASTATIC TUMOR OF THE TRICUSPID VALVE ARISING IN A CASE OF MALIGNANT TERATOMA OF THE TESTIS*

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THIS report deals with a case of teratoma of the testis in which an unusual tumor growth of the tricuspid valve of the heart was found at autopsy. The mass had produced no symptoms during life, and its presence was not suspected.

The history was briefly as follows: Seventeen months before death, the patient first noticed a swelling in the left side of the scrotum. This tumor grew rapidly, and on removal four months later was the size of an orange. The pathologic report on the specimen was teratomatous tumor of the testis.

Three months later he was readmitted to the hospital with a large mass in the left side of his abdomen. A laparotomy was performed and a large inoperable tumor the size of a grapefruit was found. Sections taken for biopsy were diagnosed secondary teratoma of the abdominal lymph glands. Microscopic sections showed the tumor to consist of a hemorrhagic and necrotic fibrous stroma containing early cartilage cells, cysts, and glands. The lining of the glands was of a low columnar epithelium, while some cysts were lined by a low cuboidal epithelium and others by stratified squamous epithelium.

Following discharge from the hospital, the patient's condition became rapidly worse, and on his last admission three months later, he complained of shortness of breath, pain in the right chest, weakness, and loss of weight. Physical examination revealed almost complete loss of resonance over the right chest, tracheal displacement to the left, clubbing of fingers and toes, and a large abdominal tumor.

At autopsy large tumor masses were found in the abdominal and pleural cavities. These tissues were irregular, soft, and hemorrhagic with many cystic areas scattered throughout the fibrous stroma. The microscopic picture was as above described.

Attached to the tricuspid valve were a number of curious, irregular masses made up of about 20 cystic structures (see Fig. 1). This tumor growth was 6 cm. in length and was firmly attached to the valve cusps, and with each of its cystic structures pedunculated and waving in the blood stream. Most of the tumor consisted of small yellowish white, firm, thick-walled cysts about the size of a match head joined together by fibrous connective tissue. There were also two large thin-walled cysts situated at either end of the mass. These cysts were soft, fluctuant, filled with a clear yellowish, watery fluid and measured 1.3 cm. in diameter.

*From the Department of Pathology, University of Toronto and Toronto General Hospital. Received for publication, June 27, 1933.

The principal tissue of the tumor was composed of a pale staining dense fibrous connective tissue. In some areas it was very compact and acellular, while in others, the wavy fibers were rich in deep staining nuclei, tending to be fusiform in shape.

The tumor masses were covered by a single layer of endothelial cells similar in appearance to those of the normal endocardium. Small thrombus masses

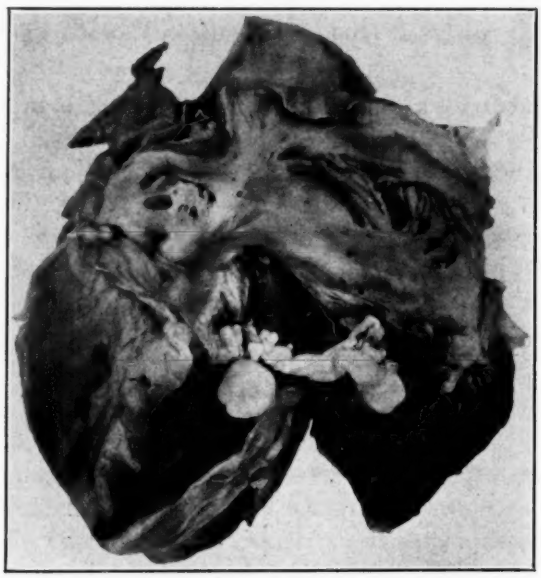

Fig. 1.—Gross appearance of cystic tumor of tricuspid valve.

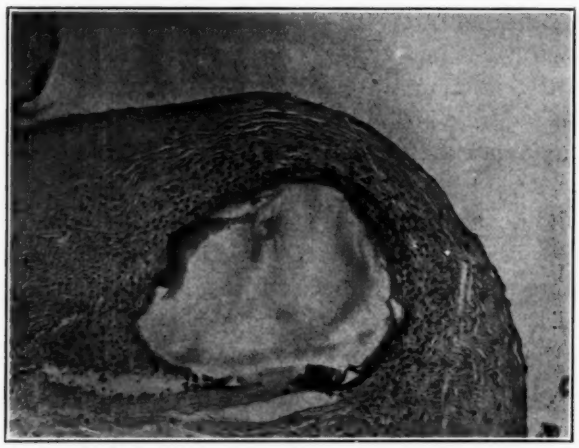

Fig. 2.—Microscopic section of epithelial lined cyst from tricuspid valve.

were found attached to the surface of the tumor. These consisted chiefly of a loose fibrin meshwork, containing many platelets and leucocytes. In a few areas the endothelial covering of the tumor was broken, and the thrombus was adherent to the fibrous tissue. The tissues were quite vascular.

The sections of an individual mass revealed several small cystic spaces. The lining of these cysts was variable in nature. One was lined in part by a

single layer of low cuboidal epithelial cells and partially by what appeared to be squamous epithelium. The cavity was filled by a small mass of desquamated epithelium. The fibrous connective tissue about the cyst was more dense and cellular than in other areas. One cystic cavity was completely lined by stratified squamous cells. This lining varied in thickness from five to fifteen cells, the upper layers being only slightly flattened. The basement membrane of the epithelium was very poorly defined. Occasionally the epithelium was of columnar character. There were also a few small clumps of cells scattered throughout the sections which presented the picture of early mesenchymal cells, showing a tendency to differentiate into cartilage.

SUMMARY

A tumor metastasis upon tricuspid valve is reported, the primary tumor being a malignant teratoma of the testis. The sections examined presented (1) a somewhat acellular fibrous connective tissue stroma, containing large areas of mucoid degeneration covered by a single layer of endothelium upon which were many thrombi; (2) the tissue contained cysts lined by both cuboidal and stratified types of epithelial cells; (3) there were present small capillary spaces filled by red blood cells; (4) small areas of mesenchymal cells suggestive of early cartilage formation were also present.

THE CAUSATIVE ORGANISMS OF PNEUMONIA IN CHILDREN IN EGYPT*

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THIS work has been undertaken to find out the common causative organisms of pneumonia in children in Egypt for which purpose specimens taken directly by lung puncture at that part which clinically showed most physical signs were sent to the laboratory. The investigation was carried out during the three months from October to December, when pneumonias are most common in Egypt, particularly in the middle part of that period.

Fifty-two cases were available. Thirty-seven were out-patients (25 males and 12 females) and fifteen in-patients (10 males and 5 females). The ages varied from four months to five years.

Thirty-five patients exhibited lobular distribution (8 right, 11 left, and 16 bilateral). Ten were of the lobar type and 6 probably so (10 right and 6 left); one case was of the pseudolobar type (right). Of the former, one was associated with an empyema (syn-pneumonic), two with slight pleural effusion, and one with meningitis probably secondary to the lung condition. Of the lobar cases 3 were complicated with a syn-pneumonic empyema. The disease was primary in 31 cases, definitely secondary to whooping cough in 2, and prob-

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Received for publication, January 17, 1933.

ably so in more, and secondary to infections of the upper respiratory tract in 14. In one patient the disease was of a tuberculous origin as verified by postmortem examination, although during life no evidence of tuberculosis was present.

The majority of out-patient cases could not be traced satisfactorily; two of the patients are known to have been cured. Of the in-patients 12 were cured, one improved, and 3 (one of them tuberculous) died.

EXPERIMENTAL

The specimens obtained by lung puncture and sent to the laboratory in sterile test tubes were plated out without delay on plates of blood agar and Fildes' peptic blood digest medium, incubated at 37° C. and examined after twenty-four hours and thereafter. To the remaining fluid in the test tubes broth was added, taking the necessary precautions to prevent contamination, and the tubes were likewise incubated at 37° C., so that in case the plated specimens proved sterile, there would still be hope of finding organisms by resorting to the broth cultures. These broth cultures, however, were only resorted to in very few instances, as it was possible in the great majority to obtain growth by primary cultivation. The colonies on these plates were examined, and pure cultures were made. Whenever necessary, biochemical and serologic reactions and pathogenicity tests were performed in order to determine the exact nature of the organism. With such organisms as frankly hemolytic streptococci and staphylococci, obviously such procedure was unnecessary, the appearance of the colonies and the films being quite sufficient. For the differentiation between Pfeiffer's bacillus and Bordet-Gengou's bacillus, the culture characteristics were largely depended upon, the better growth of the former on Fildes' medium and of the latter on Bordet's medium, etc. Sera were not available. Without producing all the protocols, it will be enough to mention that out of the 52 specimens sent for bacteriologic examination, 31 specimens were found to contain one kind of organism, 17 more than one, and 4 were sterile. The results are shown in Tables I and II.

TABLE I

CASES SHOWING THE PRESENCE OF ONE KIND OF ORGANISM. 31 SPECIMENS IN ALL

NO. OF SPECIMENS	NATURE OF ORGANISM	PER CENT
8	Pneumococcus	16
5	Hemolytic streptococcus	10
7	Nonhemolytic	14
2	Staphylococcus	4
5	Pfeiffer's bacillus	10
4	Bordet-Gengou's bacillus	8

It will be seen that out of 52 cases, pneumococci were present in 13, hemolytic streptococci in 9, and nonhemolytic streptococci in 11 cases, either alone or in association with other organisms, and that these are evidently responsible for the great majority of cases.

As typing of pneumococci from cases of pneumonias was to our knowledge not done in Egypt, owing to the relative infrequency of pneumonias in adults and the difficulty of obtaining a reasonable number of strains to work with, it

TABLE II

CASES SHOWING THE PRESENCE OF MORE THAN ONE KIND OF ORGANISM
17 SPECIMENS IN ALL

NO. OF SPECIMENS	NATURE OF ORGANISMS	PER CENT
2	Pneumococcus and staphylococcus	4
1	Pneumococcus and N. catarrhalis	2
1	Pneumococcus and Friedlander's bacillus	2
1	Pneumococcus and M. tetragenus	2
2	Hemolytic streptococcus and staphylococcus	4
1	Hemolytic streptococcus and diphtheroids	2
2	Nonhemolytic streptococcus and staphylococcus	4
2	Nonhemolytic streptococcus and B. Pfeiffer	4
1	Staphylococcus and B. Pfeiffer	2
1	Staphylococcus and N. catarrhalis	2
1	Staphylococcus and Bordet-Gengou's bacillus	2
1	Staphylococcus and Friedlander's bacillus	2
1	B. Pfeiffer, hemolytic streptococcus and an aerobic terminal spore bearer (probably a contamination)	2

TABLE III

TYPE OF DISEASE	CAUSATIVE ORGANISM	NO. OF CASES
Definitely lobar 11 cases	Pneumococcus	4
	Pneumococcus and B. Friedlander	1
	Hemolytic streptococcus	2
	Hemolytic streptococcus and diphtheroids	1
	Pneumococcus and M. tetragenus	1
	Staphylococcus and N. catarrhalis	1
	H. influenzae	1
? Lobar 6 cases	Hemolytic streptococcus and staphylococcus	1
	B. influenzae	3
	Sterile	1
	Bordet-Gengou's bacillus	1
Lobular 35 cases	Sterile	3
	Pneumococcus	4
	Hemolytic streptococcus, H. influenzae and spore bearer	1
	Pneumococcus and N. catarrhalis	1
	Pneumococcus and staphylococcus	2
	Hemolytic streptococcus	3
	Bordet Gengou's bacillus	3
	Nonhemolytic streptococcus	7
	Nonhemolytic streptococcus and staphylococcus	2
	Staphylococcus	2
	Staphylococcus and Hemophylus influenzae	1
	H. influenzae	1
	Nonhemolytic streptococcus and H. influenzae	2
	Hemolytic streptococcus and staphylococcus	1
	Staphylococcus and Bordet-Gengou's bacillus	1
	Staphylococcus and B. Friedlander	1

was thought advisable to do typing on the few available strains of pneumococci isolated from our cases. For typing, sera sent us by courtesy of the Rockefeller Institute were used and for the preparation of emulsions pure cultures on serum agar were employed. The serial method of dilution of serum was adopted, and the mixtures incubated in a water-bath at 50° C. for four hours. The result is shown in Table IV.

TABLE IV

TYPES OF PNEUMOCOCCUS	NO. OF SPECIMENS	PER CENT
Type I	2 cases	15
Type II	3 cases	24
Type III	2 cases	15
Group IV	6 cases	46

It must be admitted that the number of strains is hardly sufficient to come to any conclusion as to the distribution of types of pneumococci isolated from cases of pneumonia in Egypt. It is our intention to proceed with the collection of more strains in order to be able to give more reliable results, but the figures as they are will give an approximate idea about the distribution of the various types.

SUMMARY AND CONCLUSIONS

1. Fifty-two cases of pneumonias occurring in infancy and childhood in Egypt were examined, 35 being of the lobular and 17 of the lobar type.

2. The disease was primary as far as the history and examination showed in 3 cases and secondary in the remainder, being secondary apparently to whooping cough in 6, and to infections of the upper respiratory tract in 14.

3. In 31 instances a single kind of organism was found by lung puncture (59.6 per cent); more than one kind of organism were found in 17 cases (32.7 per cent), and the puncture was sterile (7.5 per cent) in 4 instances.

4. Altogether pneumococci were present in 13 cases (25 per cent) and streptococci in 20 cases (38.5 per cent) (hemolytic in 9 and nonhemolytic in 11), either alone or in association with other organisms. These organisms seem to be responsible for most of the cases examined.

5. Of the cases showing one kind of organism, pneumococci were found in 8, streptococci in 12 (hemolytic in 5, nonhemolytic in 7), staphylococci in 2, Pfeiffers bacillus in 5, and Bordet-Gengou's bacillus in 4 cases.

6. Typing of the pneumococci met with showed 15 per cent of Type I, 24 per cent of Type II, 15 per cent of Type III, and 46 per cent of Group IV, but the limited number of cases prevents us from considering these percentages as final.

We wish to express our gratitude to Dr. I. Shawky and Dr. A. Khalil of the Pediatric Department, Faculty of Medicine, Cairo, for their valuable suggestions.

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LABORATORY METHODS

A NEW MICROSCOPE ADAPTER FOR THE HAND SPECTROSCOPE*

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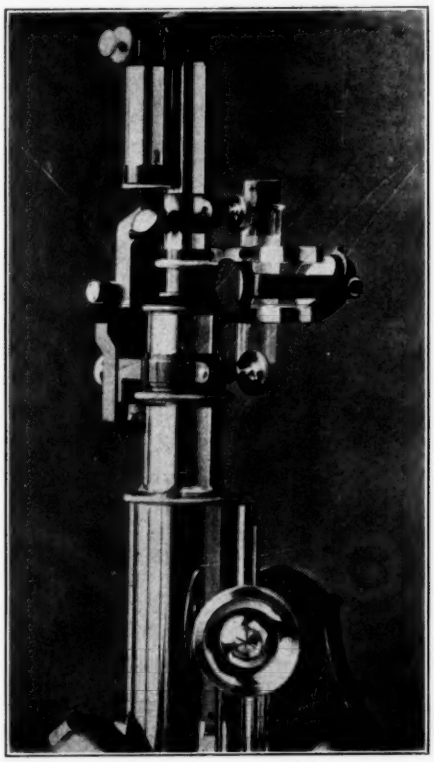
IN A recent communication I† described a method for the routine examination of blood stains in medicolegal cases. The method requires spectroscopic examination of suitably treated material on a microscopic slide, and this involves the insertion of the body of a hand spectroscope into the tube of the microscope.



I found that it was not easy to hold the spectroscope steady and at the same time make an exact reading of the wave length scale. I discussed the difficulty with the representative of Messrs. Zeiss & Co. and described to him more or less what was wanted, and the attachment presently described is the result of

*From the Bacteriological Institute, Medical Department.
Received for publication, May 5, 1933.

†Greaves, A. V.: The Use of Takayama's Solution in the Identification of Blood Stains, Brit. M. J. 2: 932, 1932.

the leaven of my suggestion working on the dough of their ingenuity. Reference to the figure will show that the body of the spectroscope is grasped by a circular band tightened by a screw with a milled head. This is connected with a heavily built standard, which in turn, is connected by a similar band attachment to the draw tube of the microscope. The spectroscope is thus held rigidly in perfect alignment with the optical system of the microscope. The standard is broken in the middle by a hinge by means of which the spectroscope may be swung away from the observer and thus free use of the ocular permitted. In the illustration may also be seen an attachment with reflecting mirror for use with a comparison prism if the spectroscope is so equipped. This may be removed if desired. The smaller tube containing the wave length scale is also equipped with a small reflecting mirror by means of which a beam of light may be passed up the tube to illuminate the scale. In the photograph the microscopic ocular is shown in place; I have found that it is much better to remove it before observing the spectrum. The method suggested for use of the appliance is as follows: The attachment (which may conveniently be left permanently applied to the spectroscope) is slipped over the tube of the microscope (after removal of the ocular) and the screw on the band tightened, so that the end of the spectroscope tube is just clear of the tube of the microscope. The hinge of the holder is adjusted so as to be on the side of the tube away from the observer. The microscope is now placed accurately in relation to the lighting system and the small mirror at the end of the tube bearing the wave length scale is adjusted so as to illuminate the scale. When these preliminary preparations have been made, the spectroscope may be swung away from the observer and the ocular replaced in the tube and the microscope is then ready for use as soon as the slide preparation is made. When the color change indicative of the presence of blood is observed on the slide (after treatment with Takayama's solution), the ocular is withdrawn, the spectroscope is swung into place and the spectrum observed and the bands located in relation to the scale. The appliance is heavily built, and there appears to be nothing about it which can get out of order or become worn.

A CENTRIFUGE METHOD FOR THE DETERMINATION OF THE VOLUME OF CELLS IN BLOOD*

GEORGE M. GUEST, M.D., AND VINTON E. SILER, A.B., CINCINNATI, OHIO

IN RECENT years increasing attention has been paid to the fact that significant changes of the volume of cells in the blood may be observed in a wide variety of pathologic conditions, and that in certain diseases the determination of these changes may afford valuable diagnostic aid. Knowledge of the relative volume of cells, of the red cell count and of the concentration of hemoglobin in a sample of whole blood permits the calculation of two values which are increasingly used in the classification of different types of anemia; namely, the average size of the erythrocytes in cubic microns and the concentration of hemoglobin per unit of volume in the red cells. Reviews of most of the reported studies of this subject may be found in articles by Haden (1931, 1932), Osgood, Haskins, and Trotman (1932), and Wintrobe (1932). The determination of the relative volume of cells in blood also permits the indirect estimation of various chemical constituents of the cells by calculation from determinations made on whole blood and plasma in circumstances where direct chemical study of the cells is less satisfactory.

Numerous methods have been offered for the determination of blood cell volume, but most of them have certain features which present more or less serious obstacles to their general application. Ponder and Saslow (1930) have reviewed many of the methods and discussed the faults inherent in each type of procedure. These authors claimed that their modification of the Stewart (1899) colorimetric method was the most accurate means of determining cell volume, but this method is extremely tedious and the amount of blood required is prohibitive if the bloods of infants or small laboratory animals are to be studied. Although the centrifuge method in its many modifications has been criticized by Ponder and Saslow as well as by others who have studied the problem, this procedure is, nevertheless, generally considered to be the most practical for clinical use. Aside from other advantages, the hematocrit tube has one distinctive point of superiority over other devices, in that it offers the only practical means of measuring separately the total cell volume and the red cell volume. This is important if the cell volume value is used to calculate the size of the erythrocytes or the hemoglobin content of the red cells, since the failure to exclude the large white cells may often introduce into these calculations an easily perceptible error.

Judging by the published reports of cell volume studies, the Van Allen (1925) method appears to be one of the most popular of the several procedures in current clinical use. The Van Allen tube is convenient and is economical of

*From the Children's Hospital Research Foundation and the Department of Pediatrics, College of Medicine, University of Cincinnati.

Received for publication, May 24, 1933.

blood, but it has one potential source of error in common with all types of hematocrit tubes with which rubber sealing devices are used; such sealing devices occasionally allow slight leakage which may pass unnoticed, and this danger is increased the higher the centrifuge speed to which the tubes are subjected. Another objection to this method is that the diluting fluid (1.3 per cent sodium oxalate solution) while perhaps isotonic for normal blood, may not necessarily be isotonic for pathologic blood samples in which osmotic changes have occurred. This criticism is applicable to all those methods employing anticoagulant salts which may cause changes of cell volume. Whether a supposedly isotonic oxalate solution is used as in the Van Allen (1925) and Haden (1923, 1925) methods, or whether dry oxalate is used and correction made for shrinkage of the cells, as in the Osgood (1926) and Wintrobe (1929) methods; in either case the constants determined for normal bloods have not been proved dependable for bloods in which osmotic changes have occurred under different pathologic conditions.

Economy of blood is nearly always an important consideration and at times is essential in these studies. Haden (1930) maintained that 10 c.c. of blood—the amount used in his method—was not an excessive amount to take from a patient. This is perhaps true of adults, but it is usually impractical to remove such an amount from infants, especially when more blood is to be drawn for other chemical studies. In discussing various methods Haden also claimed that a macromethod was necessary for accuracy, but in our own experience it has been possible to make closer measurements in capillary tubes than in larger tubes. It is, moreover, easier to differentiate the layer of white cells if the columns of cells are measured in capillary tubes.

Gram and Norgaard (1923) employed a capillary tube with heavy walls, 10 cm. in length and graduated in 200 intervals, with hirudin as anticoagulant. Later Rosahn (1931) devised a tube somewhat similar to that of Gram and Norgaard and used heparin as the anticoagulant. In both procedures the tubes were sealed with rubber bands for centrifuging.

In several methods employing capillary tubes the use of rubber sealing devices has been avoided. Smirk (1929) used thin-walled capillary tubes of 0.3 mm. bore, bent into U-shape. Oxalated blood was drawn into such tubes with a layer of paraffin oil at each end of the column of blood to diminish the loss of CO_2, and the tubes were centrifuged at 3,000 to 4,000 r.p.m. until the cells were packed to constant volume. The lengths of the columns of cells and plasma were then marked on paper by pricking points, the distances between the points measured with dividers, and the cell volume in percentage calculated from these measurements. The results from the two columns of the U-tube were averaged. Smirk claimed that, at the same speed of rotation, more complete separation of plasma from cells was obtained by centrifugation in capillary tubes than in larger tubes, and that separation was more complete in shorter columns of blood, 5 to 6 cm., than in longer columns. Bönniger (1909) also had employed a U-shaped tube. Campbell (1922) used thin-walled capillary tubes which were partly filled with oxalated blood by dipping one end into the blood, and the opposite end then sealed in a flame. After centrifugation the lengths of the columns of cells and plasma were measured by a millimeter scale and the

percentage cell volume calculated. Epstein (1915) used heavier capillary tubes, of 0.5 to 1.0 mm. bore, with one end drawn out to be sealed in a flame. He placed granules of heparin in the tube and used blood obtained by skin puncture. After the tube was partly filled with blood and the small end sealed, the blood column was measured end to end and the thin end of the tube inserted into a cork for centrifugation. After centrifugation the length of the column of plasma was measured, the plasma volume was calculated in percentage and the volume of cells obtained by difference.

The method for the determination of the relative volume of cells in blood which is to be described here has been developed after a considerable amount of experimental work with many of the reported centrifuge methods; and various steps in the procedure have been adopted, with modifications, from a number of different sources. Briefly: in this method thin-walled capillary tubes in pairs are partly filled with heparinized blood, one end sealed in a flame, and the tubes thus filled are centrifuged until the cells are packed to constant volume. The columns of cells and plasma are then measured by means of a measuring microscope, and from these measurements the volume of "total" cells and "R.B.C." are calculated in percentage of whole blood. A high speed centrifuge with special head and a special measuring microscope are recommended, but practically identical results may be obtained with the ordinary types of laboratory centrifuge and microscope if the latter be equipped with a cross-hair eyepiece and a graduated mechanical stage. The principal advantages of the method are that it is economical of blood; that the anticoagulant used apparently does not affect the size of the cells; that the volumes of red cells and white cells may be read separately with ease; that errors due to rubber sealing devices are avoided; that it is rapid.

APPARATUS

Vials to receive the blood samples may be prepared, a large number at a time, by placing in small vials appropriate amounts of a 1 per cent solution of heparin* in distilled water. Convenient sizes are 2 c.c. or 4 c.c. vials which receive respectively 0.1 or 0.2 c.c. of this solution; i.e., 1.0 or 2.0 mg. of heparin. The water is then evaporated from the vials in a drying oven at 100° C., or in a vacuum oven at a lower temperature. In either case the vials should not be left in the oven longer than necessary, because after prolonged heating the heparin tends to dissolve in the blood less readily. When dry, a glass bead and a closely fitting rubber stopper are placed in each vial.

Capillary tubes are prepared from ordinary soft glass tubing (internal diameter 10 mm. and wall 1.0 mm. in thickness) which has been cleansed with acid cleaning mixture, rinsed with distilled water and dried. This tubing is heated over a Bunsen burner with wing top adjusted to give as even a flame as possible, and the heated portion is drawn out 15 or 20 feet by two people walking rapidly away from each other. The pulled capillary tube should be held taut until well cooled, and then dropped onto a flat surface in order to

*The heparin is obtainable from Hynson, Westcott and Dunning, Baltimore. Several writers have suggested the advantages of the use of heparin in cell volume studies, but dismissed it as too expensive. The use of small blood samples reduces the cost, and in this method of dispensing there is little waste.

minimize its tendency to warp into curves. From this capillary tubing 12.0 cm. lengths are cut and carefully selected for straightness and absence of taper. Such selection may be made conveniently by rolling the capillaries on a flat surface (plate glass) which is slanted a few degrees. The curved tubes are culled out by their failure to roll, and a significant degree of taper will cause the tubes to roll to one side. The most suitable capillary tubes may range in internal diameter from 0.4 to 0.8 mm., with walls 0.1 to 0.15 in thickness. In our experience the yield of acceptable tubes prepared in this fashion is from 20 to 40 per cent. Because the heat employed in drawing the tubing may cause some alkali to appear on the surface of the glass, the selected capillaries should be dropped into acid, rinsed with water and dried. Such rinsing is easily done by placing a number of the capillary tubes in a glass cylinder or test tube which is filled and emptied several times in succession with acid, distilled water, alcohol, and ether. After the final washing with ether, the capillaries may be placed in a drying oven for a short time to drive out the ether.

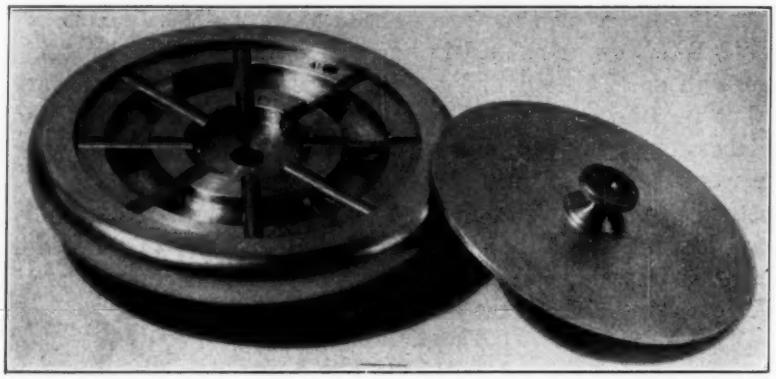


Fig. 1.—Centrifuge head used with the high speed attachment of the International centrifuge.

The labor involved in preparing the capillary tubes is somewhat compensated by the fact that the tubes when used once are thrown away, and no time is spent cleaning them.

Centrifuge.—The special head shown in Fig. 1 may be used for centrifuging the capillary tubes on the high speed attachment of the International centrifuge.* This head has a radius of 75 mm., and has places for eight small metal cylinders, each bored to carry a pair of 6 cm. capillary glass tubes. The tubes are carried horizontally, in their plane of motion, so that at the end of centrifuging the tops of the columns of packed cells are level. With this head the best results have been obtained by a speed of 14,000 r.p.m. for from four to five minutes. An automatic timing device to stop the centrifuge is helpful.

For centrifuging in the usual style cups with an ordinary type of centrifuge capable of speed around 3,000 r.p.m., carriers for the capillary tubes can be made from a block of wood or aluminum made to fit loosely in a centrifuge cup and bored with small holes, each hole just large enough to carry a pair of the capillary tubes. Several pairs can be carried in one such block.

*The International Equipment Co., Boston, Mass.

Measuring Microscopes.—In all the control studies listed here the measurements of the columns of blood cells and plasma after centrifuging were made by means of the measuring microscope shown in Fig. 2.* The positions of the microscope, the light source, etc., as shown in the figure, are arranged for the comfort of the operator. Two pieces of opaque paper are pasted on a plate of glass so as to make a horizontal narrow slit in which the capillary tube is placed for the measurements. The light is reflected from a mirror through the slit and readings are made by transmitted light which makes the line of demarcation between white and red cells more distinct. While this microscope permits readings to 0.01 mm., any laboratory microscope (low power objective) equipped with a cross-hair eyepiece and a graduated mechanical stage with vernier which permits readings to 0.1 mm. gives results practically identical

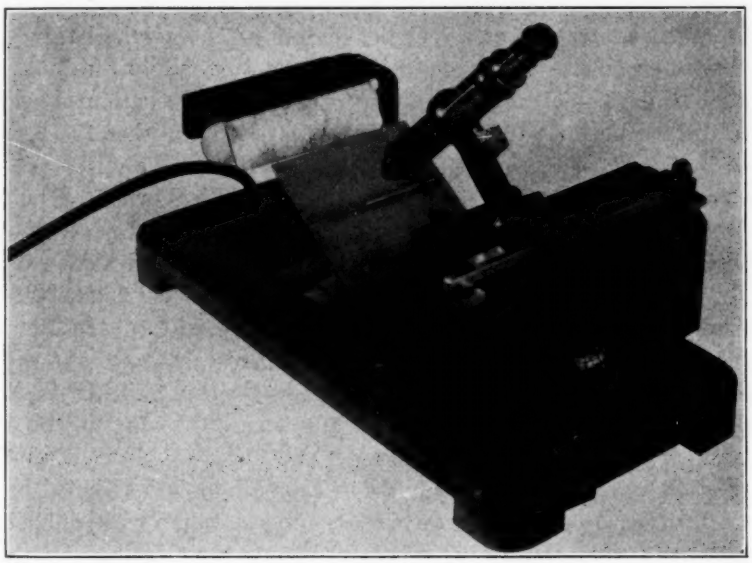


Fig. 2.—The measuring microscope, arranged for measuring the lengths of columns of blood cells and plasma.

with those obtained by closer readings. For use with the ordinary microscope, a holder for the capillary tubes may be made by pasting two pieces of opaque paper on a glass slide so as to make a narrow slit in which the capillary tube is placed during its measurement. Light is reflected through this slit from below by the mirror of the microscope substage.

METHODS

The blood sample for the cell volume determination should be obtained with a dry syringe and needle and quickly mixed with the heparin, 2 c.c. of blood to 1.0 mg. of the anticoagulant, in a small vial such as previously described. Care should be observed to avoid air bubbles in the blood discharged into the vial, and mixing of the anticoagulant should be by gentle inversion of

*Obtained from the Central Scientific Company, Chicago, Illinois.

the tube without violent shaking which is likely to produce foaming. If tissue juices are mixed with the blood, or if slight clotting of the blood starts in the syringe before it is discharged into the heparin vial the formation of particles of fibrin may cause difficulty even though the blood appears grossly to be unclotted. With slight fibrin formation the line of demarcation between the red and white cells is harder to read and sometimes the white cells are found scattered through the red cells, apparently caught in the strands of fibrin. The heparinized blood may be used also for cell counts, hemoglobin estimation and any other chemical examinations with which heparin does not interfere.

At the time of use one of the 12.0 cm. capillary tubes is cut in the middle to give a pair of tubes which are turned to bring their "outer" ends together. Several pairs (usually four in our own work) should be used for each blood sample. The "outer" ends of each pair of tubes are thrust deeply into the *well mixed* blood and filled by capillarity to within about 2.0 cm. of the "inner ends"—i.e., the ends that came from the middle of the original tube. Finally a layer of paraffin oil 2.0 to 3.0 mm. deep may be drawn into the tubes over the columns of blood. The oil serves mainly to prevent evaporation of water from the surface of the plasma; it is doubtful whether it diminishes the loss of CO_2, as claimed by Smirk who used it in a similar fashion. The oil is not essential to secure results sufficiently accurate for routine clinical work. The "inner" ends are then sealed by holding them perpendicular to and just touching the outer margin of a micro burner gas flame. With care and slow rotation the tube can be sealed quickly with a flat inner base. It is important that this be done carefully, since slight taper in the sealed base of the tube makes the final measurement of the lengths of the blood column less accurate. If the wall of the tube is too thick, or if the glass has not a low melting point, it is difficult to make such a seal quickly and the glass may become hot so far up the tube that there is danger of overheating the lower part of the blood column. This manner of sealing excludes the possibility of even slight loss of blood by leakage which is a frequent source of error in hematocrit tubes with rubber sealing devices. The advantage of having the pair of tubes cut from one longer tube, and turned as described, lies in the fact that the slight taper usually present even in the most carefully selected capillaries is then in the opposite direction in each tube of the pair, and, therefore, the error due to taper in the one compensates that of the other in the final results.

The tubes thus filled and sealed are centrifuged, either in the special head (Fig. 1) at approximately 14,000 r.p.m. for from four to five minutes, or in the ordinary centrifuge at approximately 3,000 for thirty minutes. In samples of dog's blood with red cell counts as high as eight million or more, the period of centrifuging at 14,000 r.p.m. should be extended to five or six minutes. With slower speeds a longer time is necessary to completely pack the cells; with speeds higher than 14,000 r.p.m. the red cells in some blood samples tend to rupture and the hemoglobin becomes diffused into the layer of white cells, making the line of demarcation between the red and white cells harder to read. After centrifuging, the capillary tubes are immediately placed upright in a block of wood bored with small holes to receive them; if left lying horizontally for more than a few minutes the top of the layer of cells will be slanted.

For the measurements, each capillary tube is placed in the light slit of the glass slide before the measuring microscope. With the stage of the latter set at zero, the capillary tube is adjusted by hand to bring the base of the blood column to the cross-hair of the eyepiece; the stage is then moved laterally and readings are made at the line of demarcation between the red and white cells, at the top of the white cells and at the meniscus of the plasma. From these measurements on each tube the cell volumes, "R.B.C." and "Total," are calculated in percentage of whole blood. The values for each pair of tubes are averaged (as shown in Table I), and as a rule the maximum variations between these averages in a set of four pairs of tubes is less than 0.4 per cent.

CONTROL STUDIES

Internal Diameter of the Capillary Tubes.—Two sets of capillary tubes were selected, the capillaries in each set having internal diameters 0.3 to 0.4 mm. and 0.6 to 0.8 mm., respectively. Six pairs in each set were filled alike, sealed in a flame as described and centrifuged at 12,000 r.p.m. for four minutes. The averages for the total cell volumes calculated for the two sets of tubes were respectively 45.41 and 45.39 per cent. Although there was found no significant difference between the results from the two sets, practically the tubes with internal diameters from 0.5 to 0.7 mm. are the most satisfactory to use. The smaller ones are too fragile; while the larger ones are harder to seal with a good flat inner base, and the thicker blood column makes the line of demarcation between the white and red cells harder to read by transmitted light.

The Effect of Taper in the Capillary Tubes (Table I).—It was previously stated that the reason for having the pair of capillary tubes cut from one longer tube is that they may be turned so that if slight taper is present, it will be in the opposite direction in each tube of the pair and the error due to taper in one will compensate that of the other. This effect is illustrated in Table I by the readings of a set of tubes in which the differences are considerable between the members of each pair and yet the agreement is good between the averages of the separate pairs.

TABLE I

EFFECT OF TAPER IN THE CAPILLARY TUBES

READINGS IN MILLIMETERS			CALCULATED CELL VOLUMES IN PER CENT			
R.B.C.	W.B.C.	PLASMA	R.B.C.	AVERAGE OF PAIR	TOTAL	AVERAGE OF PAIR
23.60	24.13	50.80	46.46		47.50	
24.06	24.56	51.04	47.14	46.80	48.12	47.81
23.11	23.58	50.97	45.34		46.26	
25.43	25.97	52.96	48.02	46.68	49.04	47.65
23.65	24.13	51.22	46.17		47.11	
24.50	24.99	51.85	47.25	46.71	48.20	47.66
24.53	25.16	52.40	46.81		48.02	
23.99	24.59	51.41	46.66	46.74	47.83	47.93
General Average				46.7		47.8

Length of the Columns of Blood (Table II).—Pairs of capillary tubes, 6 cm. in length and from 0.4 to 0.6 mm. in internal diameter, were filled with heparinized blood sufficiently to give blood columns ranging from 20 to 55 mm. in total length. These were sealed in a flame as usual and centrifuged at 12,000 r.p.m. for four and one-half minutes. The results listed in Table II indicate that the length of the columns of blood within these limits affects the final results very little.

TABLE II

EFFECT OF LENGTH OF BLOOD COLUMNS

LENGTH OF BLOOD COLUMNS MM.	NUMBER OF PAIRS	CELL VOLUMES IN PER CENT R.B.C.	TOTAL
20-29.9	7	45.63	46.53
30-39.9	7	45.74	46.53
40-55.0	9	45.72	46.45
Total Pairs	23		
Mean		45.70 ± 0.03	46.51 ± 0.02
Standard Deviation		± 0.18 ± 0.02	± 0.16 ± 0.02
Coefficient of Variation		± 0.38% ± 0.04	± 0.35% ± 0.03

Time of Centrifuging at 14,000 r.p.m. Within a 7.5 cm. Radius (Table III).—From a sample of heparinized normal human blood, 7 sets of capillary tubes, 4 pairs in each set, were filled and sealed as described. These sets in turn were centrifuged in the special head at 14,000 r.p.m. for periods from one-half minute to ten minutes. During the one-half minute period high speed was not attained. The results shown in Table III indicate that at 14,000 r.p.m., four to five minutes is sufficient to obtain constant packing of the cells in human blood. In this centrifuge, run at this speed, periods longer than ten minutes were undesirable because if run a longer time the apparatus tended to become heated.

It may be noted here that in the capillary tubes translucency of the blood cell column (Koeppe's criterion [1905]) cannot be accepted by itself as a criterion of complete packing of the cells; a fair degree of translucency sometimes may be observed before the cells are completely reduced to constant volume. The conditions of speed and time must be adjusted to obtain this maximum packing of the cells (without rupture of the cells), but on the other hand too long periods of centrifuging should be avoided because of chemical changes which may occur in the cells and alter their volume.

TABLE III

TIME OF CENTRIFUGING AT 14,000 R.P.M.

TIME MINUTES	CELL VOLUMES IN PER CENT R.B.C.	TOTAL
½	61.88	62.75
1	48.15	49.01
2	46.85	47.59
3	46.50	47.27
4	46.45	47.31
6	46.44	47.26
10	46.51	47.25

Time of Centrifuging at 3,000 r.p.m., Within a 20 cm. Radius.—From a sample of heparinized normal human blood 5 sets of capillary tubes, 4 pairs in each set, were filled and sealed as described. Four sets were centrifuged in a 50 c.c. cup and holder, such as described under apparatus, with a No. 1 International centrifuge (Type SB) using a head with 15 cm. radius (the bottom of the cup traveled at a 20 cm. radius). When these 4 sets were centrifuged at 3,000 r.p.m. for fifteen, thirty, forty-five, and sixty minutes, the average total cell volumes measured in these sets were respectively 44.4, 44.2, 44.0, and 44.2 per cent. In the fifth set, centrifuged in the special head at 14,000 r.p.m. for four minutes, the total cell volume was 44.1 per cent.

In ordinary practice, therefore, centrifuging the capillary tubes to constant cell volume in the ordinary laboratory centrifuge should usually give results as satisfactory as those obtained with the special high speed centrifuge. In certain pathologic conditions, however, there is need to handle blood samples as quickly as possible after the blood is drawn, because rapidly progressing intracellular chemical changes may alter the size of the cells. For example, in certain types of severe acidosis and in gastroenteric intoxication of infants, it may be observed that glycolysis, lactic acid formation and liberation of inorganic phosphates by intracellular hydrolysis of organic phosphorus compounds occur more rapidly than in normal bloods (Guest [1932]). In circumstances such as these, the shorter period of centrifugation at higher speed is advantageous.

Effect of Standing at Room Temperature (Table IV).—Heparinized normal human blood was allowed to stand at room temperature (28° to 32° C.; this experiment being done in the summer) for seventeen hours. At intervals after the blood was drawn, indicated in Table IV, sets of four pairs of capillary tubes were filled, sealed, and centrifuged at 13,500 r.p.m. for four and one-half minutes. The results listed in Table IV indicate that in the first three hours there was a noteworthy shrinkage in the cell volume; part of this effect may be due to loss of CO_2 from the blood, and part to changes concomitant with glycolysis. In the later periods the increased amount of lactic acid accumulating as a result of glycolysis may be responsible for the swelling of the cells.

TABLE IV

THE EFFECT OF STANDING, AT ROOM TEMPERATURE

TIME AFTER BLOOD WAS DRAWN	CELL VOLUMES IN PER CENT	
	R.B.C.	TOTAL
15 minutes	45.8	46.4
1¼ hour	45.4	46.2
3 hours	44.6	45.4
6 hours	45.1	45.8
11 hours	46.2	47.0
17 hours	46.8	47.5

Anticoagulants (Table V).—A sample of normal human blood was drawn and delivered in 5 c.c. samples into small vials containing the following: (1) heparin 2.0 mg., (2) hirudin 2.0 mg., (3) sodium citrate 0.015 gm., (4) sodium oxa-

late 0.015 gm., (5) sodium fluoride 0.015 gm. Another sample, No. 6, was defibrinated with a glass rod. Four pairs of capillary tubes were prepared from each of these samples and centrifuged as usual. The results of the test are listed in Table V. It may be noted that identical results were obtained with the use of heparin and hirudin.

TABLE V

EFFECT OF DIFFERENT ANTICOAGULANTS

ANTICOAGULANT		CELL VOLUMES IN PER CENT R.B.C.	TOTAL
Heparin	0.04%	45.1	45.8
Hirudin	0.04%	45.1	45.8
Sodium citrate	0.3 %	41.7	42.4
Sodium oxalate	0.3 %	39.5	40.0
Sodium fluoride	0.3 %	36.3	36.8
Defibrinated	–	44.8	45.3

Amount of Heparin.—A sample of normal human blood was added to heparin in two vials in the proportion 0.5 mg. per c.c. and 2.0 mg. per c.c., respectively. Eight pairs of capillary tubes were filled from each vial and centrifuged at 14,000 r.p.m. for four minutes. The mean values for total cell volume in the two sets were respectively 41.92 ±0.11 and 41.84 ±0.11 per cent; two values with no significant difference by mathematical test. It may be assumed, therefore, that between these limits the proportion of heparin added to blood need not be accurately measured.

Types of Glass Used in the Capillary Tubes.—Because it is easier to draw capillary tubing from lead glass and to obtain a high yield of straight capillary tubes, it was hoped that this type of glass could be used. However, when sets of lead glass and soda lime glass capillary tubes were used for the same blood samples, the lead glass tubes gave results for cell volume about 2 volumes per cent lower than those obtained with the soda lime glass tubes. When these capillary tubes were kept for twenty-four hours after the determinations, it was observed that the packed cells in the lead glass capillary tubes become increasingly opaque, whereas, in the soda lime glass tubes the column of cells remained translucent. It seems likely that the lower cell volume was due to the effect of lead entering the blood from the glass. Pyrex glass is much more difficult to draw into capillary tubing sufficiently uniform for the needs of this method, and it is more difficult to seal Pyrex glass capillaries properly without overheating the blood in the tube.

Comparison of Cell Volume Determinations by Other Methods.—Twenty-five cubic centimeters of blood were drawn from a normal human adult and this sample divided for cell volume measurements according to four different methods: (1) The Sanford-Magath (1929) modification of the Haden method, (2) Van Allen method (1925), (3) Wintrobe (1929) method, (4) the capillary tube method as here described. Results averaged for several tubes used in each of these methods were respectively 45.4, 45.1, 47.9, and 47.3 per cent for the total cell volumes.

Studies of blood cell volume and erythrocyte size in arterial and venous blood, the effects of contact of the blood with air and changes in CO_2 content,

and the effects of stasis of blood in veins before blood samples are drawn, will be reported in a later communication. It may be stated here, however, that when blood samples are handled as described in the foregoing pages, the slight losses of CO_2 which may result from brief contact of the blood with air have practically no effect upon the cell volume.

SUMMARY

A method for the determination of the percentage volume of cells in blood has been presented. Small capillary glass tubes in pairs are partly filled with heparinized blood, the tubes sealed in a flame, and the tubes then centrifuged until the cells are packed to constant volume, preferably at 14,000 r.p.m. for from four to five minutes in a special centrifuge head. The lengths of the columns of cells and of plasma are read either by a special measuring microscope or by means of an ordinary microscope equipped with a graduated mechanical stage. The readings are made to the top of the red cells, to the top of the white cells, and to the top of the plasma. From these measurements the relative cell volumes "R.B.C." and "Total" are calculated in percentage of whole blood. Control studies are presented to indicate the accuracy and variations to be expected in measurements made by this method.

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A PRACTICAL METHOD FOR THE CONTINUOUS ADMINISTRATION OF FLUID INTRAVENOUSLY*

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WHEN the intravenous administration of liquid is desirable, an efficient apparatus designed to perform this service is welcome. Such an apparatus must accomplish its function with safety and dependability, as well as provide for the comfort of the recipient, if it is to compete with other methods.

The apparatus here described was designed specifically for the intravenous administration of liquids at constant rates for long periods of time. Its primary purpose is to deliver liquid into the veins of sick patients under conditions determined by them, meanwhile satisfying the requirements of asepsis, of safety, of dependability, and of ease of manipulation.

Although it will probably find its greatest uses clinically in those cases requiring long treatment, this apparatus is also well adapted for use in cases where no cooperation is offered by the recipient. In the laboratory, when mounted on a rotating platform on top of a cage, it becomes an efficient instrument for the continuous intravenous injection of animals.

The apparatus, which is shown in the photograph (Fig. 1), consists of (1) an eccentric rotor driven by a variable-speed synchronous type motor, and (2) an independent tube for conducting the liquid. The pump itself operates on the well-known principle of "milking a tube," modified to avoid all connection between pumping mechanism and conducting system. A powerful propulsion of liquid is effected by the progression of the eccentric rotor over a spiral segment of the conducting tube. The rest of the apparatus embodies several other principles which guarantee the uninterrupted injection of sterile liquid even without the cooperation of the recipient.

In order to assure a smooth and uninterrupted one-way flow, the liquid is carried under pressure to the point of discharge, and is there released to flow freely into the vein. This operation is achieved through use of a release valve at the end of the conducting tube which not only offers resistance to the forward flow, but also prevents the backward flow of liquid. The valve consists of a small piece of rubber tube which expands against a hole through which the fluid must pass. By this means the uniformity of flow is not affected by the swinging, stretching, or other manipulation of the conducting tube.

The asepsis of the injection procedure is maintained by a completely closed system, entirely independent from the pumping mechanism. This closed system

*Received for publication, May 16, 1933.

is sterilizable in the autoclave, since it consists of a single tube from beginning to end without connections of any kind.

The recipient is relieved of the duty of keeping his arm in one position by a combination of three features. The first has already been mentioned: liquid will be discharged uniformly in spite of all manipulations of the conducting tube. The second is that the tube is so firmly anchored to the arm by a substantial arm plate (Fig. 3) that it cannot be dislodged. The third is that the

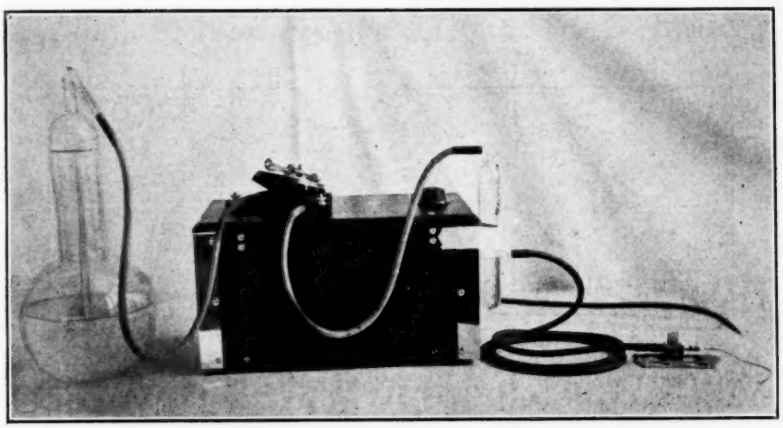

Fig. 1.—Photograph of the complete apparatus.

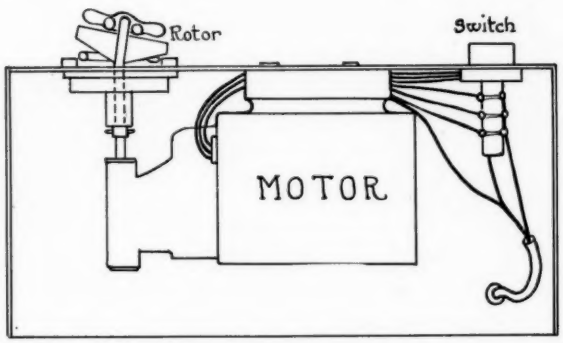

Fig. 2.—Sketch of the mechanism.

liquid is injected into the vein through a fine rubber capillary tube which is so flexible that no movements of the arm can affect its position within the vein. Not only may the recipient move his arm as he pleases, but also is only little disturbed by the capillary tube in his vein.

When the injection is prolonged and the exchange of supply flasks frequent, a connection such as that shown in Fig. 1 is useful. It consists of a glass tube to withdraw the liquid and a sealed-on glass skirt to enclose the neck of the flask. This connection insures sterility, facilitates refilling, and permits the flasks to stand upright.

If the operator wishes that a signal be given when the supply flask has been emptied to a certain level, he may set the flask upon one pan of a balance which

has a counterpoise on the other pan representing the weight of the flask when it has been emptied to this level. Further withdrawal of liquid will then cause the balance to swing and close an electric circuit containing a suitable signal. He may also interpose a trap in the conducting tube to catch air and sediment and to indicate the rate of flow.

The technic used to operate this apparatus is as follows: After sterilization, the conducting tube is attached to the machine and the machine set in motion. When all the air has been driven out of the tube and liquid has begun to flow regularly from the release valve, an intravenous needle (Size 15) is placed in

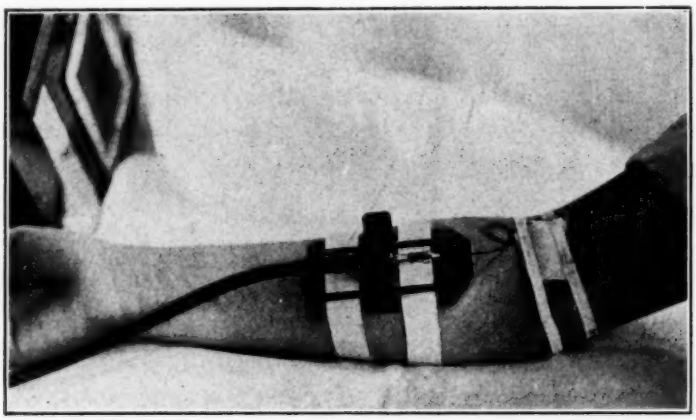

Fig. 3.—Photograph of arm of recipient with apparatus attached.

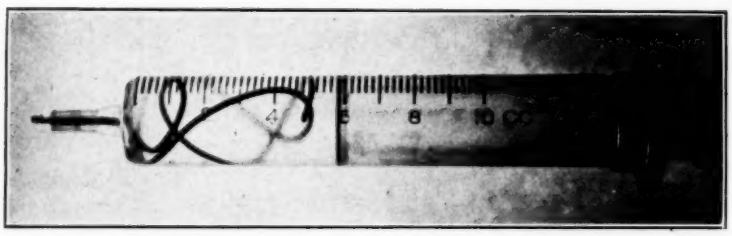

Fig. 4.—Photograph of a "threaded" syringe.

the vein. The capillary rubber tube is injected through the needle from a syringe (Fig. 3). The knot in the proximal end of the capillary tube, which was placed there to catch at the shoulder of the syringe, is cut off after the syringe has been detached, and then the needle is withdrawn. The capillary tube is attached to the release valve through a fine Luer needle. The release valve is attached to the arm plate after the latter has been taped to the arm. A small dressing is placed over the puncture wound through which the tube passes. The injection may then be continued indefinitely, the recipient meanwhile having the liberty to move his arm as he pleases. The rate of injection may be changed by the ratios of 1:2:4 by turning a switch. The present machine delivers liquid at the rates of 75, 150, and 300 c.c. an hour. Rates outside these limits require the use of tubes of suitable size.

Because the discharge into the vein is slow and constant the liquid need not be warmed. For the same reason hypertonic solutions of glucose (in normal salt solution) may be injected safely.

The several mechanical features in this apparatus which contribute to its successful performance are the following: The driving mechanism is completely enclosed and will run indefinitely without attention. The pump will develop enough pressure to burst the tube, yet the wear on the tube by the rotor is negligible. The release valve is made of stainless steel and furnishes, through connection to the arm plate, absolute anchorage. All parts susceptible to deterioration, namely, the conducting tube, the rubber capillary tube, and the tube in the release valve, are readily and cheaply replaceable. Clotting within the capillary tube is impossible, because blood has no opportunity to enter it, since liquid is continuously flowing out. A smooth and uniform discharge from the capillary tube is guaranteed by a balance of two forces, the cushioned liquid pressure within the conducting tube and the resistance of the release valve. The rubber capillary tube, which was developed especially for this work, has an outside diameter of 0.04 inch. It is made of high grade anode rubber, and it will retain its elasticity and tensile strength through many days of contact with blood.

The Oak Manufacturing Company, Chicago, Illinois, kindly undertook to make this machine.

THE STABILITY OF DIOTHANE SOLUTIONS*

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ATTENTION has already[1] been called to the unusual behavior of some phenyl urethane local anesthetics when their solutions are boiled. The monophenyl urethanes of di-n-butyl- and di-iso-butyl-amino propanediols were found to be altered by boiling their solutions in such a way that their activity as surface anesthetics materially increased while the anesthesia produced by their intradermal injection was shortened. This was interpreted as being due to some change of an unknown nature which resulted in the creation of a more rapidly diffusible form of anesthetic.

When diothane (piperidinopropanediol di-phenyl urethane hydrochloride) was selected as a new and valuable anesthetic for clinical trial, the behavior of its solutions on heating was checked, and it was found and reported that sterilization over prolonged periods did not alter the solution according to physiologic tests.[2]

A further detailed chemical study of diothane and its solutions has revealed the fact that the anesthetic free base is relatively unstable to heat. Heat-

*From the Research Laboratories of The Wm. S. Merrell Company.
Received for publication, May 23, 1933.

ing this free base to its decomposition point in a vacuum has yielded traces of aniline together with other unknown decomposition products. The crystalline hydrochloride is quite stable to moderate heat (100° C.) when the crystals are dry, but somewhat unstable to heat when they are wet with water or other solvent capable of partially dissolving them. The amount of change is too slight to allow the isolation of any decomposition products.

Since the wet crystals proved to be somewhat unstable it was deemed advisable to restudy the stability of diothane solutions.

The treatment of local anesthetic solutions with dilute nitrous acid followed by the addition of an alkaline solution of beta-naphthol is a recognized test for distinguishing those products containing primary amino groups. Thus procaine gives a dark brick-red precipitate (positive) while cocaine gives a yellow-white precipitate (negative). Freshly prepared solutions of pure diothane crystals give a typical negative reaction. However, when these solutions are heated for prolonged periods (> one hr. at 98° C.) or aged for many months, diazotization and coupling with beta-naphthol gives a light orange precipitate.

This change as attested by the delicate chemical reaction is accompanied by no demonstrable change in anesthetic activity or toxicity of the solution, indicating that the extent of any change is extremely slight.

Theoretical consideration of possible changes which would result in the creation of diazotizable amino groups suggested only two reactions. The first of these was a possible rearrangement as follows:

$$<\!\underline{\quad}\!> NH - COO - \rightarrow NH_2 <\!\underline{\quad}\!> COO - \text{ (or isomer)}$$

Since this rearrangement would account for the formation of a colored dye in the test reaction, an attempt was made to determine the amount of change which would account for the degree of color actually developed. In order to check this point to some extent diothane solutions were made up containing various quantities of procaine, and these solutions were tested together with the oldest diothane solution available (two years). The colors developed by the solution containing procaine were quite different in shade, but the intensity of color developed by the old diothane solution was similar to that of a fresh diothane solution containing procaine 1:90,000. Similar tests were carried out by adding traces of the di-p-amino benzoate homologues of diothane, and since these products contain two primary amino groups per molecule even smaller proportions were required to give colors of the same intensity.

In this connection it is of interest that the di-p-amino benzoate of piperidinopropanediol[3] has almost no activity as a mucous membrane anesthetic. It is obvious, therefore, that if this product were found in diothane solutions in any quantity, the change would be detectable by physiologic tests.

Remembering that thermal decomposition of diothane free base had yielded traces of aniline, diothane solutions with added quantities of aniline hydrochloride were also tested in comparison with the oldest diothane solution. Again the shade of color developed in the test was somewhat different, but an even closer comparison of intensities was possible. In this case, the intensity de-

veloped with the old diothane solution was most closely matched by that with a fresh diothane solution to which aniline had been added in a concentration of 1:200,000.

In both cases the color developed by the diothane solution, to which the primary amino compounds had been added, was concentrated on the otherwise yellow-white precipitate, making the very slight color formation quite obvious. In neither case was there similarity enough in the shade of color to indicate that either an amino benzoate or aniline had actually been formed. These experiments indicate, however, that an extremely small proportion of the diothane had been altered.

While in the above discussion the two-year-old diothane solution was specifically mentioned, the same alteration of the solution may be rapidly achieved by heating the solution for several hours on a steam-bath. The addition of a slight excess of acid to the solution markedly delays such changes as take place, and no significant change occurs during the first hour of heating, so that solutions may be adequately sterilized without alteration.

Attempts to correlate the changes in diothane solutions with the increased surface activity of the di-n-butyl- and di-iso-butyl-amino propanediol monophenyl urethane solutions have not been productive, since these boiled solutions do not yield any colored product on attempted diazotization and coupling with beta naphthol.

SUMMARY

Prolonged heating or aging of diothane solutions produces a very slight degree of alteration detectable by delicate colorimetric testing, but not detectable by pharmacologic methods. Theoretical considerations of possible changes have been discussed, and it has been inferentially shown that the concentration of any alteration product in diothane solutions is probably not greater than 1:100,000.

The writer wishes to acknowledge the assistance of Dr. E. W. Scott and Messrs. A. R. Lux and Karl Bambach.

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THE PRESERVATION OF BIOLOGIC SPECIMENS*

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TISSUES which are to be preserved for study or exhibit are often fixed by means of a formalin solution, and then kept immersed in an aqueous solution of sodium acetate containing glycerin and thymol. It is a common observation that the preserving media become clouded after a few weeks or months and must be replaced. To reduce the necessity for frequent replacement, we have undertaken a study of the cause of clouding, hoping to be able to control it by suitable means.

Our investigations have shown the turbidity, which seems not to be peculiar to any special kind of tissue although some specimens induce more rapid clouding than others, is due to oxidation of the thymol in the preserving fluid. While thymol is an inhibitor of bacterial growth, its solubility in water is low, and there seemed to be a possibility of bacterial changes causing precipitation in our solutions. This possibility has been definitely disproved as will be shown later.

METHODS OF STUDY

In these studies, we have used the solutions already referred to. Tissues known by experience to have a tendency to cause clouding were fixed by soaking in a solution of the following composition: Chloral hydrate 500 gm., sodium chloride 180 gm., sodium bicarbonate 100 gm., sodium sulphate 220 gm., 40 per cent formalin solution 500 c.c., water to make 10,000 c.c. After the tissues had been hardened in this solution for several days, they were washed in running water for twenty-four hours, and then, after suitable mounting, kept immersed in a solution consisting of glycerine 2,000 c.c., sodium acetate 800 gm., thymol 100 gm., and water to make 10,000 c.c. The thymol was dissolved in boiling water, added to the main bulk of solution, and the whole cooled and filtered before using. Considerable excess thymol was removed in this step. Where special solutions were needed, they were made up in small amounts, and their use is reported below.

The tissues used for our controlled experiments were from a normal brain, and showed a marked tendency to induce clouding.

Since clouding of our media did not set in normally until six weeks or more after they had been prepared, we heated some of our specimens to a temperature of about 50° C., so that precipitation would be observed in a matter of days rather than weeks. The conclusions to be drawn from the experiments at the higher temperatures can with due allowance be applied to conditions at room temperature.

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Received for publication, May 19, 1933.

RESULTS AND INTERPRETATION

The first clue to the cause of clouding was found in the observation that all specimens led to turbidity, and that where it occurred the turbidity began after several weeks' standing and then increased slowly to a maximum. The white cloudiness seemed to be of the same character in all cases and resembled that noted when saturated thymol solutions are cooled.

It was thought that some obscure bacterial infection was the cause of the difficulty. Were there bacterial action operating, however, the clouding would probably develop quickly and continuously. In an endeavor to observe the actual organisms present, clouded fluid was examined under a microscope. It appeared perfectly clear. This indicates that the precipitate is very finely divided, but does not exclude the possibility of very small microorganisms being present. All efforts to culture the clouded media failed. Inoculation of a new sample of the preserving fluid with clouded material failed to accelerate the development of new cloudiness. These observations indicated the absence of bacterial factors in this phenomenon.

It became evident to us that some slow chemical change in the medium itself, perhaps influenced by material extracted from the biologic specimens, is responsible. Two simple observations convinced us that this is the case, and that the source of the clouding is the thymol in the preserving medium. First, a badly clouded solution in which a panophthalmia exhibit had been kept was clarified by repeated boiling with paper fibers, and filtration. It was returned to the original jar containing the specimen and showed no further precipitation over a period of four months. On adding thymol to this solution, slight clouding was noted after four weeks. Second, a sample of the usual medium alone showed a turbidity after standing in the laboratory for from six to eight weeks.

The precipitate, we noted, is insoluble in acid and alkali, but is quickly dissolved by ether. It is not coagulated by strong calcium chloride solution. This behavior conforms to the expected action of thymol or a thymol derivative. The precipitate, we noted further, always begins at the surface of the preserving solution, indicating oxidation as a possible factor. This was confirmed in the following way. A sample of the original medium was slightly diluted with water and kept at 50° C. A second sample was simultaneously diluted with hydrogen peroxide solution, and kept at the same temperature. The latter sample clouded in a few hours, the former did not. It appeared, therefore, that oxidation has some part in the phenomenon. The experiment was repeated at room temperature. The medium with peroxide clouded in four days, the control medium has remained clear for as many weeks.

To determine whether the biologic samples themselves contribute to this oxidation, we kept a sample of tissue under water at 50° C., for several days. Naturally there was considerable extraction of soluble material in this period. The water extract was filtered and added to some preserving medium. A precipitate developed in a matter of a few days, where otherwise, it would not. Some water soluble material from the tissues, therefore, must catalyze the formation of the observed precipitate. Whether there may be other reactions than oxidation of the thymol which contribute to the clouding, our experiments do not show. We believe, however, that this is likely.

AN IMPROVED PRESERVING FLUID

While these observations have identified thymol as the cause of precipitation in our fluids, there remained the problem of finding an adequate substitute. Certainly replacement of the thymol seems called for. A tissue placed in preserving medium without thymol has remained indefinitely clear, but there is some danger of mould growth in an acetate solution not protected by a fungicide. We have found that a small amount of phenol or toluol added to the solution gives the desired protection. Such solutions have remained clear for a period of nearly six months.

We have tried other methods of preservation, most of which have proved unsatisfactory. A specimen kept under pure glycerin became rather badly discolored after a short time. A thin coating of collodion did not prevent clouding of one specimen, but a coating of varnish did. Had the collodion film been thick and air-dried more thoroughly, it would undoubtedly have further slowed up the clouding, but would not eventually preclude its appearance. The coating of varnish, likewise, does not offer permanent protection against clouding.

A layer of paraffin oil over the acetate preserving solution, by slowing up the admission of oxygen, inhibited but has not prevented clouding of the medium. When immersed in pure paraffin oil and maintained at 50° C. for several weeks, one specimen caused no clouding, but on cooling there was developed a heavy turbidity, which disappears on reheating. This probably is due to water which was distilled into the paraffin at the higher temperature, and which was thrown down by cooling. At constant temperature, however, there is no precipitate and if kept at room temperature, paraffin oil should be a satisfactory preserving medium. By actual test, we have found the oil with tissue immersed in it to remain clear for over three months. Substitution of magnesium sulphate in equivalent concentration for the sodium acetate does not greatly retard the formation of cloudiness; it had been thought that the higher ionic charge would decrease the formation of the colloidal suspension.

SUMMARY AND CONCLUSIONS

1. An investigation of the media in which biologic specimens are preserved shows that the frequently observed clouding is due to a chemical oxidation and precipitation of thymol in the preserving fluid, catalyzed by materials extracted from the tissues.

2. An improved fluid in which phenol or toluol is substituted for thymol has proved satisfactory in practice.

3. Paraffin oil may be used as a preserving fluid, provided the temperature does not vary over too wide a range.

The assistance of Dr. M. J. Fein, pathologist of the Cumberland Hospital, in carrying out this work is acknowledged.

CHANGES IN CELL VOLUME PRODUCED BY VARYING CONCENTRATIONS OF DIFFERENT ANTICOAGULANTS*

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A STUDY of the distribution of certain inorganic elements in cells and plasma reveals that the cell-plasma ratio varies not only with the nature of the anticoagulant but also with its concentration. Since the accuracy of such an investigation is dependent upon a definite cell volume as a basis for each calculation, the literature was investigated to ascertain the preferable method of procedure. Many substances are recommmended as anticoagulants, the kinds, concentrations, and manner of use being almost as variable as the number of investigators. Definite lengths of time and speed of contrifuging, as a rule, are not stated. Guillaumin,[1] Eisenman,[2] and Hirota[3] all give some data pertaining to the subject in general but fail to furnish information applicable to this problem. In many cases, apparently, the manner of determining the cell volume was largely a matter of choice, so far as any definite method of procedure was concerned. For these reasons, it was deemed advisable to investigate the effects of the commonly recommended anticoagulants, and the conditions most suitable for determining the true cell volume.

EXPERIMENTAL

Of the anticoagulants commonly recommended, the following have been employed in this investigation; sodium, potassium, and ammonium oxalates of concentrations 0.1 to 0.4 per cent by weight, the corresponding citrate salts of concentrations 0.2 to 0.8 per cent by weight, and heparin of concentrations 0.1 to 1 mg. per ml. of blood.

The cell volumes were determined using 4 mm. bore hematocrit tubes 15 cm. in length. The cell and serum heights in the tubes were measured with vernier calipers, thus eliminating the use of graduated tubes. The tubes were filled by means of capillary tubing; after which they were sealed and suspended on a rubber cushion in a position which would bring their axes perpendicular to the axis of rotation while centrifuging. A large size standard International Centrifuge was used, the speed being checked by use of a tachometer.

The time required at various speeds for the cell volume to become constant was ascertained. Determinations were made at two speeds—1800 and 2200 R.P.M. At lower speeds the time required was excessive, and it was difficult to prevent hemolysis; at higher speeds the tubes were frequently broken. The apparent cell volume was read at intervals during the centrifuging, and the data so obtained are presented in the accompanying chart. An examination of these curves reveals that in the case of cow's blood the cell volume becomes constant in 65 minutes at 1800 R.P.M. and in 45 minutes at

*Received for publication, June 21, 1933.

2200 R.P.M. while for chicken blood, which contains larger cells, only 40 minutes at 1800 R.P.M. were required.

Using these standard conditions the cell volumes were determined with the various anticoagulants previously mentioned. Blood of cattle, chickens, and rats was drawn with an hypodermic needle, either from the jugular vein or by heart puncture, and delivered into flasks coated with the designated quantity of anticoagulant. After thorough mixing and chilling, the blood was delivered to the hematocrit tubes as described. The concentrations mentioned earlier in this article represent the extremes used by most investigators. These tests have been frequently repeated for various types of blood, and typical results are shown in Table I.

It will be observed from an inspection of the table that the ammonium salts produce an increase in cell volume with an increase in the percentage of

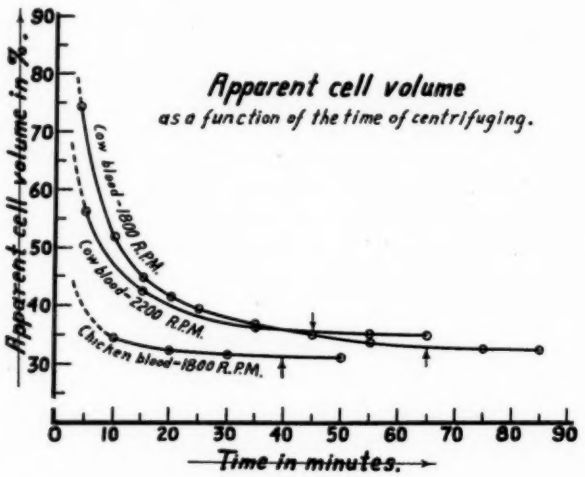

Chart 1

anticoagulant used, while sodium, potassium, and lithium salts produce a cell-volume decrease with percentage increase of sats used. Various determinations indicate that about 0.2 per cent of the oxalates and 0.4 per cent of the citrates produce the most favorable results from the standpoints of anticoagulation and hemolysis. Since ammonium salts produce an increase in cell volume and the other salts have an opposite effect, various mixtures have been tested, and it has been found that with the use of an 0.2 per cent concentration of a mixture of 40 per cent potassium oxalate and 60 per cent ammonium oxalate there is a minimum change in cell volume. These data are presented in Table II.

In the analysis for inorganic constituents, any inorganic anticoagulant interferes. Heparin was tried for this reason although it carries a calcium contamination. Its use is more difficult than that of the inorganic compounds as it does not dissolve as readily due to its dryness, and there is, consequently, a tendency to form small clots about the particles of heparin. It cannot be dissolved and evaporated over the surface of the container as it is an organic

TABLE I

CHANGES IN CELL VOLUME WITH VARYING CONCENTRATIONS OF ANTICOAGULANTS

OXALATES				CITRATES			
ANTICOAGULANT	CELL VOLUME		SOURCE OF BLOOD	ANTICOAGULANT	CELL VOLUME		SOURCE OF BLOOD
	AMOUNT PER CENT	CHANGE			AMOUNT PER CENT	CHANGE	
Potassium oxalate			Cow	Potassium citrate			Rat
0.1%	33.33			0.2%	34.20		
0.2%	32.20	−1.13		0.4%	32.60	−1.60	
0.3%	31.02	−1.18		0.6%	31.20	−1.40	
0.4%	30.22	−0.80		0.8%	30.10	−1.10	
Sodium oxalate			Cow	Lithium citrate			Cow
0.1%				0.2%	32.33		
0.2%	32.42			0.4%	31.20	−1.33	
0.3%	31.22	−1.20		0.6%	29.85	−1.35	
0.4%	29.70	−1.52		0.8%	29.10	−0.75	
Ammonium oxalate			Chicken	Ammonium citrate			Chicken
0.1%	31.34			0.2%	28.81		
0.2%	32.65	+1.31		0.4%	29.85	+1.04	
0.3%	33.30	+0.75		0.6%	31.98	+2.13	
0.4%	33.80	+0.50		0.8%	33.97	+1.99	
Ammonium oxalate			Cow				
0.1%	39.66						
0.2%	40.82	+1.16					
0.3%	41.54	+0.72					
0.4%	41.80	+0.26					

TABLE II

THE EFFECT OF A MIXTURE OF POTASSIUM OXALATE AND AMMONIUM OXALATE ON CELL VOLUME

ANTICOAGULANT	CELL VOLUME		SOURCE OF BLOOD
	AMOUNT PER CENT	CHANGE	
40% Potassium oxalate and 60% Ammonium oxalate			Rat
0.1%	41.2		
0.2%	41.2	0	
0.3%	41.1	−0.1	
0.4%	41.0	−0.1	

compound, which might decompose on heating. The most satisfactory procedure consisted in making a water suspension, which caused the heparin to dissolve rapidly when added to the blood. Although the use of this compound as an anticoagulant has been repeatedly tried, it is impossible to secure results that check as favorably as those for the inorganic compounds. Table III presents the averages of two series of results from the experimental use of heparin to coagulate the blood of rats and of chickens. These data indicate

TABLE III

CELL VOLUME PRODUCED BY VARYING CONCENTRATIONS OF HEPARIN AS COMPARED TO POTASSIUM OXALATE

(MG. PER ML.)

SOURCE OF BLOOD	$K_2C_2O_4$	HEPARIN			
	2	0.1	0.2	0.4	1.0
Rat	45.22	44.28	43.70	42.00	
Chicken	27.00	26.19	26.19	26.16	26.37

that the concentration does not affect the ratio noticeably, but there is some cell packing with greater concentration. The cell volume is always slightly less than when inorganic anticoagulants are used.

CONCLUSIONS

1. There is a need for a specified concentration of anticoagulant as well as speed and time of centrifuging if cell-serum ratios are to be used in calculations.

2. Increasing concentrations of sodium, potassium, or lithium salts cause a decrease in cell volume.

3. Ammonium salts cause an increase in cell volume with increased concentration.

4. Within normal limits, a mixture of 40 per cent potassium oxalate and 60 per cent ammonium oxalate gives a minimum cell volume variation.

5. A speed of 2200 revolutions per minute was found most satisfactory, all factors being considered.

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THE KOLMER, KAHN, AND MEINICKE REACTIONS IN THE TROPICS*

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THE object of this study was to adopt for our laboratories a test which can be rapidly performed, is of highest sensitivity, is of greatest technical accuracy, and which is specific in its reaction, uniform in results, and also simple and economical.

MATERIAL

1. Serums from 700 syphilitic cases included primary, secondary, and tertiary congenital and latent syphilis. One-third of the cases presented secondary syphilis. Most of the patients were untreated.

2. Serums of 300 nonsyphilitic controls were obtained from 100 patients with severe secondary anemia due to intestinal parasites; from fifty cases of acute malarial fever; from fifty cases of chronic malaria; from twenty-five cases of mycotic disease of the skin; from 10 lepra cases; fifteen frambesia and fifty postoperative cases. None of these patients presented any symptoms or gave any history of syphilis. Careful observations were made on all cases; complete physical examinations and histories were taken in all.

*From the Laboratories of the Santa Clara Hospital and Departments of Pathology and Parasitology of the University of Cartagena.

Received for publication, June 29, 1933.

TABLE I

RESULTS OBTAINED AND METHODS USED IN 700 CASES OF SYPHILIS

METHODS	NUMBER OF POSITIVE REACTIONS	NUMBER OF DOUBTFUL REACTIONS	NUMBER OF NEGATIVE REACTIONS
Kolmer Complement Fixation	515	5	180
Kahn Standard Precipitation	525	15	160
Meinicke Clarification	520	12	168

TABLE II

RESULTS OBTAINED IN 300 NONSYPHILITIC CONTROLS

NUMBER OF CASES	NAME OF DISEASE	KAHN		KOLMER		MEINICKE	
		POSITIVE	DOUBTFUL	POSITIVE	DOUBTFUL	POSITIVE	DOUBTFUL
		+	+−	+	+−	+	+−
100	Secondary Anemia of Parasitic Origin	2	4	0	0	2	5
50	Acute Malaria	1	2	0	1	1	4
50	Chronic Malaria	1	2	0	0	1	2
25	Mycosis of Skin	0	2	0	0	1	3
10	Lepra	1	1	0	1	1	2
50	Postoperative Cases	1	2	0	0	4	4
15	Frambesia	15	0	15	0	15	0
	Total	6	13	0	2	10	20

All methods gave 100 per cent positives in frambesia

COMMENT

The Kolmer complement fixation test is harder to perform and less economical than the precipitation tests of Kahn and Meinicke, but the results obtained are of greater uniformity and give the lowest percentage of nonspecific doubtful reactions. In our group of negative serums, we had not one nonspecific positive reaction with the Kolmer test. Even though it is a more complicated test to learn and harder for the technician to perform, yet once mastered, the test is much more easily interpreted than the precipitation tests. It gives clear negatives in every type of the tropical diseases in which we have used it, when there was no manifestation of syphilis involved. In the repeated tests of the same serums the Kolmer test gave the same reactions.

The Kahn test, though of greatest sensitivity, is less specific in reaction than the complement fixation test. However, the simplicity of the test and the rapidity with which it can be done recommend it for daily routine in the laboratories of tropical countries. The Kahn test is as hard to interpret as it is easy to perform. It requires skill and good eyesight to avoid false reactions. Doubtful reactions should always he repeated in conjunction with the complement fixation test. This test is of great value in emergencies as an immediate aid in the diagnosis.

The Meinicke test is less sensitive than the Kahn and gives the greatest percentage of nonspecific positive reactions in our group. It is very easy to perform and the interpretation, when delayed for several hours, is much easier

than in the Kahn test. When read immediately, the same difficulties are met. Our technicians prefer the Meinicke test to other precipitation tests because of the easier interpretation.

In all laboratories the precipitation tests should be always performed in conjunction with the complement fixation test. In the tropics, a precipitation test is of great value because of the rapidity with which it can be performed. The climate tends to decompose the blood very rapidly, and often it is necessary to perform the test as soon as specimens reach the laboratory. All doubtful and suspected positive reactions are always repeated in conjunction with the complement fixation tests. For specificity, we depend on the Kolmer test.

CONCLUSIONS

1. One thousand serums have been studied by the Kolmer, Kahn and Meinicke reactions; 700 serums were from known syphilitic cases, and 300 were negative controls.

2. The precipitation tests were found most sensitive, but less specific than the complement fixation test.

3. The precipitation tests are less complicated to perform and more economical, but they are harder to interpret than the complement fixation test.

4. The precipitation tests are readily adaptable in routine laboratory procedure; because of the rapidity with which the test may be performed, it is of value in emergencies.

5. The Kolmer complement fixation test is more complicated to perform, but easiest to interpret. It is most specific in reaction.

6. The precipitation tests should always be used in conjunction with the complement fixation test, except in emergency cases.

I am grateful to Dr. Kolmer and Dr. Kahn for supplying me with antigens for this study.

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NOTE ON THE DETERMINATION OF BLOOD UREA BY DIRECT NESSLERIZATION OF A SODIUM TUNGSTATE—SULPHOSALICYLIC ACID FILTRATE*

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THERE are several methods of determining urea by direct nesslerization of a protein-free or nearly protein-free blood filtrate. Karr,[2] Roe and Irish,[5] Myers,[4] and Looney[3] all have described methods based on the nesslerization of a Folin and Wu[1] tungstic acid filtrate of blood. None of these methods has been entirely satisfactory for routine procedures. The aeration method of Myers[4] is simple and, if relatively large numbers of determinations are being run simultaneously, is very satisfactory. If only an occasional urea determination is made, or if speed is of importance, either the aeration method or the distillation method requires too much time.

Woodward and Fry[6] have recently used sulphosalicylic acid for the preparation of an acid, protein-free blood filtrate for the determination of blood glutathione. This reagent seemed to offer possibilities as a protein precipitant for the direct determination of urea in blood.

EXPERIMENTAL

Woodward and Fry used one volume of an approximately molar solution of sulphosalicylic acid for the precipitation of the proteins of one volume of blood after dilution with eight volumes of water. They found that clear protein-free solutions could be obtained with human blood. In the experiments reported here, it has been found that some bloods, particularly swine blood, gave a brownish colored filtrate when sulphosalicylic acid alone was used. Other bloods usually gave clear, colorless filtrates. By including sodium tungstate in the precipitating mixture, the yield of filtrate was smaller but, with all bloods examined, a colorless filtrate was obtained. These filtrates were found to develop a brownish color when made alkaline to litmus. The further addition of alkali resulted in a perfectly clear, colorless solution. The amount of alkali in 2 ml. of a dilute solution of Bock-Benedict Nessler's reagent was found to discharge completely the brown color of 5 ml. of neutralized blood filtrate. Nesslerized solutions, with or without ammonia present, would remain clear for several hours.

Procedure for the Determination of Urea in Blood.—

Solutions:

1 M sulphosalicylic acid; 25 gm. of sulphosalicylic acid in 100 ml. of solution (Woodward and Fry[6]).

10 per cent sodium tungstate solution (Folin and Wu[1]).

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Received for publication June 27, 1933.

Urease solution of Folin and Wu mixed just before using with the activating phosphate solution in a ratio of 9:1.

Bock-Benedict Nessler's solution (Myers[4]).

Nitrogen standard. 0.0472 gm. $(NH_4)_2SO_4$ per liter, 5 ml. 0.5 mg.

Sodium hydroxide approximately 2/3 N.

Technic:

Two milliliters of oxalated blood (2-3 mg. of potassium oxalate per milliliter of blood) and 1 ml. of fresh urease solution are pipetted into a large test tube or a small Erlenmeyer flask, the contents mixed, and the container stoppered and placed in a water-bath at 50 degrees C. for fifteen minutes. To the tube is added 13 ml. of distilled water, 2 ml. of sodium tungstate solution and with constant agitation, 2 ml. of the sulphosalicylic acid solution. The material is then filtered through a fairly retentive filter paper.

Five milliliters of filtrate is pipetted into a 25-ml. graduate cylinder. If a high urea content is anticipated, 5 ml. or more of water may be added; otherwise 1 ml. of ⅔ N sodium hydroxide is pipetted into the solution followed immediately by 2 ml. of dilute Nessler's reagent. The solution may be diluted further to approach the color intensity of 0.5 mg. of ammonia nitrogen in the standard.

The calculation then is:

$$\frac{S}{R} \times 0.5 \times \frac{D}{100} \times \frac{100}{0.5}$$

TABLE I

RECOVERY OF ADDED UREA

SAMPLE	MG. UREA N ADDED	MG. UREA N FOUND	PERCENTAGE RECOVERY
2 ml. blood	0.000	0.107	----
2 ml. blood	0.194	0.300	99.4
2 ml. blood	0.388	0.500	101.3

TABLE II

COMPARISON OF THE SSA DIRECT AND AERATION METHODS OF DETERMINING UREA NITROGEN

SAMPLE	SPECIES	MG. PERCENTAGE N		PERCENTAGE DIFFERENCE
		AERATION	SSA	
1	Swine	12.2	11.8	–3.3
2	Swine	15.0	15.0	0.0
3	Swine	16.3	15.7	–3.6
4	Swine	11.6	12.5	7.8
5	Swine	11.9	12.3	3.3
6	Swine	10.6	10.8	1.8
7	Ox	10.4	10.0	–3.8
8	Ox	13.0	13.5	3.9
9	Ox	12.0	12.0	0.0
10	Horse	8.5	8.6	1.2
11	Horse	9.0	9.1	1.1
12	Horse	7.4	7.5	1.4
13	Sheep	17.3	16.7	–3.4
14	Dog	10.7	11.3	5.6
15	Dog	9.5	9.0	–5.2
16	Human	13.5	13.0	–3.7
	Average	11.8	11.8	0.0

Effect of the Sodium Salt of Sulphosalicylic Acid on Nesslerized Solution.—Two nitrogen standards containing 1 mg. N were prepared; to one was added a neutralized solution of 5 ml. of M sulphosalicylic acid. After nesslerization and dilution to 100 ml., the two solutions gave identical intensity of color in the colorimeter.

Recovery of Added Urea.—Aliquot portions of a blood sample were measured into tubes. Water was added to the control, and urea solution to the other two tubes in order to keep all volumes equal. The results are shown in Table I.

Blood urea nitrogen of various species of animals was determined by the direct method as described, and simultaneous determinations were made by the aeration method. These samples were obtained from different sources, and nothing is known regarding the health of the animals. The comparative data are shown in Table II.

DISCUSSION

In the precipitation of the proteins of blood and urease, it has been found necessary to use both sodium tungstate and sulphosalicylic acid in order to get a colorless filtrate satisfactory for nesslerization. The filtrate obtained by using sulphosalicylic acid only was sometimes very brown, having almost the color of a nesslerized solution. Although this color was discharged by making the solution decidedly alkaline, the use of the sulphosalicylic acid and sodium tungstate combination was found preferable.

The alkalinity of the final solution is very important. Too high a concentration of alkali results in a turbid solution. It would be satisfactory to add the sodium hydroxide to the Nessler solution provided that sufficient amounts of acid were also added to the standard. Satisfactory results have been obtained by adding the sodium hydroxide after the Nessler's solution, instead of before as previously stated.

Fowl blood does not lend itself to the determination of urea by this method. The blood is low in urea, and it is very difficult to obtain samples free of clots. Cloudy solutions, which may be due, at least in part, to the high concentration of oxalate necessary to prevent coagulation, have frequently resulted.

Woodward and Fry[6] state that too much oxalate prevents complete precipitation of proteins with sulphosalicylic acid. The same has been found for citrates and fluorides; however, the amounts generally used as anticoagulants cause no difficulties.

SUMMARY

A simple method for the direct determination of urea in blood has been described.

ADDENDUM

Because of unavoidable circumstances the paper of Taylor and Blair[7] was overlooked during the experimental work reported in the preceding paper. A few analyses have been made in comparing the two methods on pooled samples of swine blood. The results indicate that either method is satisfac-

tory provided that the colorimetric comparisons are made soon after nesslerization. Nesslerized solutions of the Folin-Wu filtrate frequently develop turbidity on standing for fifteen minutes or more. This turbidity does not develop in the nesslerized sodium tungstate sulphosalicylic acid filtrates. From the point of view of simplicity of preparation, the alcoholic urease is more easily prepared and is equally as effective as the concentrated urease in the volume of blood used for analysis.

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SIMPLIFYING THE MECHANICS OF BACTERIAL FILTRATION*†

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NUMEROUS articles have appeared in medical and scientific periodicals upon various aspects of filters which remove bacteria from fluids. An extended bibliography is found at the end of a chapter by Mudd in Rivers' volume on Filterable Viruses.[1] The worker who uses filtration as a frequent and important procedure in his work must necessarily familiarize himself with this literature and will gather together many hints and suggestions in regard to the mechanics of the process. On the other hand, the worker who seldom uses filtration usually fails to take time to read so widely and often learns the technic by trial and error. Many times some slight error in the arrangement of the apparatus or some accident in the process of filtering the sample will make it necessary to repeat the operation before a sterile filtrate is obtained. Because of this fact it seemed worth while to put into print a brief discussion of the most frequent pitfalls of the procedure.

Although more convenient types of apparatus for certain purposes have been devised, such as that described by Mudd,[2] the most frequently used arrangement is filtration through a Berkefeld or Mandler candle, or a Seitz disc, into a flask with a side arm, and the suggestions given here will deal with that simple set-up.

*From the Massachusetts Department of Public Health and the Harvard School of Public Health.

Received for publication, June 28, 1933.

†The laboratory facilities for carrying on an investigation involving filtration are being provided through the courtesy of Dr. W. G. Smillie.

One cause of difficulties often results from the attempt to filter without a manometer to determine the pressure. It is easy to exceed the critical pressure of the candle and draw bacteria through. A manometer is not expensive, and one like that illustrated in Fig. 1 (*A*) can usually be assembled in a laboratory without having to purchase anything except the mercury and perhaps a piece of tubing with a 1 mm. lumen. The principle of the manom-

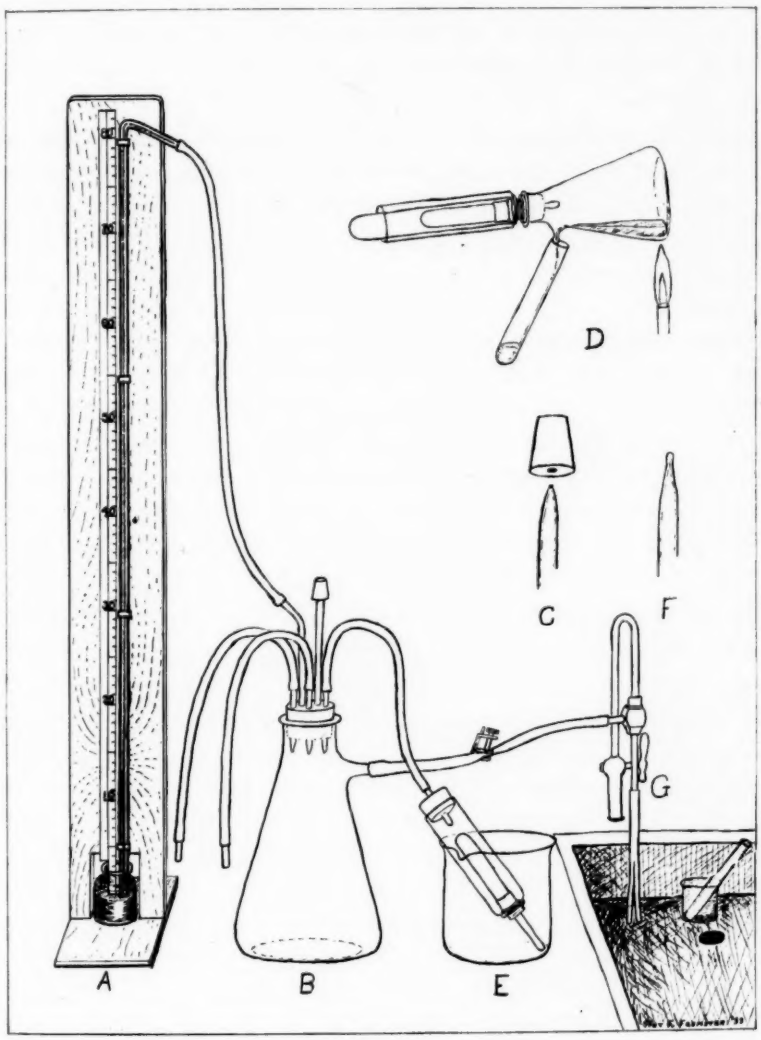

Fig. 1

eter is very simple. It consists essentially of a glass tube standing in a bottle of mercury. Attached to the upper end of the tube is a piece of rubber tubing connected with the flask which is interposed between the water pump and the flask receiving the filtrate. A support for the tube and a metric rule completes the apparatus. The latter should touch the surface of the mercury. It is a good rule to do the filtration at as low a pressure as possible. Most

filtrates can be put through with sufficient rapidity at from 100 to 150 mm. of mercury. When the Berkefeld N filter candles are used, the pressure should never exceed 450 mm. of mercury, and bacteria will sometimes come through at that pressure.

The amount of water going through the vacuum pump should be just sufficient to maintain the pressure at the desired level. A pinchcock on the tubing, Fig. 1 (*B*), connecting the pump with the filtering flask aids in controlling the pressure and is particularly useful in eliminating the effect of sharp, quick changes in pressure in the water pipes. The orifice left open should be the smallest which will not cause the mercury to drop.

A very annoying thing, which sometimes occurs, is to have the pressure suddenly released when the pump is turned off and to see the cotton plug in the side arm forced into the flask. Refiltration is, of course, necessary since the filtrate is no longer sterile. Such accidents can be avoided by having a valve which will gradually release the pressure. A very simple type of valve is illustrated in Fig. 1 (*B*) and details are shown in Fig. 1 (*C*). It consists of a piece of glass tubing which extends through a rubber cork of an intervening flask placed between the water pump and the flask which receives the filtrate. The end of the tube is drawn out and rounded off in a flame until the opening is only about a millimeter in diameter. Over this is pushed a soft rubber stopper in which a hole has been pierced about two-thirds of the distance from the bottom to the top. The tightness of the rubber stopper around the outside of the glass tube prevents the entrance of the air until it is pulled off at the end of the filtration simultaneously with turning off the pump. Air gradually goes in through the small orifice, and the cotton plug in the side arm is not disturbed.

When small quantities are to be filtered, time can be saved and loss due to liquid remaining at the bottom of the mantel can be avoided by inverting a test tube over the filter candle. The tube should be of as small a diameter as possible to still go down to the bottom of the mantel. It should be placed over the candle before the liquid is poured into the mantel. If placed on after the candle is wet, the air is often never entirely exhausted from the inside of the tube, and the fluid level never rises.

Occasionally an accident occurs when taking the rubber stopper from the flask in order to remove the filtrate, particularly if a few drops of fluid happen to fall outside the mantel and down on the stopper. This difficulty can be overcome by removing the filtrate through the side arm, provided care has been taken that no fluid has run down the rubber tube into the side arm and has dampened the cotton. The cotton is removed from the side arm and the end carefully flamed. It is then held over the end of the tube or flask which is to receive the filtrate, as shown in Fig. 1 (*D*). A flame is applied to the bottom of the flask so that the heating of the air inside causes it to expand and forces the filtrate out. Without the heat the filtrate will not run out of the side arm. When flame is being applied to the side arm, care should be observed not to overheat it, as the liquid will then cause it to crack.

Washing the candles is simple when the arrangement shown in Fig. 1 (*E*) is used. The washing liquid placed in the beaker is pulled through the candle in the reversed direction. The only equipment necessary is a stopper through which a piece of glass tubing passes. The stopper fits snugly into the end of the mantel and the glass tubing is connected with the water pump.

More than one filter can be washed at the same time with the same pump when the arrangement illustrated in Fig. 1 (*B*) is used. Each rubber tube can be connected with a filter, either when filtering or when washing the filters. When only one tube is being used, the other tubes must be closed by a pinch-cock or by thrusting a piece of glass tubing closed at one end, illustrated in Fig. 1 (*F*), into the end of each rubber tube.

Splashing in the sink by the outflow of the water pump can be avoided by the use of a piece of rubber tubing which reaches to one-quarter of an inch of the bottom of the sink. Splitting the lower three or four inches of the tube also reduces the splashing (Fig. 1 *G*).

SUMMARY

The various aspects of the mechanics of bacterial filtration are discussed, methods of eliminating the frequent difficulties, which make refiltration necessary, being particularly considered.

Four useful additions to the usual technic are described:

1. A method of using or washing three or more filters simultaneously with the same pump.
2. An easily constructed vacuum release.
3. A simple method of removing the filtrate from the flask.
4. A means of avoiding splashing by the outflow of the water pump.

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DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

B. TUBERCULOSIS, Viability and Virulence of Old Cultures of, Corper, H. J., and Cohn, M. L. Am. Rev. Tubere. 28: 856, 1933.

In 1919 and 1920, several hundred 8 ounce nursing bottles containing about 3 ounces of 5 per cent glycerol-broth, neutral to litmus, were planted with 19 different strains of human and bovine tubercle bacilli, and were maintained at 37° C. from then until 1932, when 47 bottles containing cultures of human tubercle bacilli were chosen to determine the viability of the bacilli. Twenty (42.5 per cent) of these were found capable of growing on an inspissated egg yolk medium, while, from 9 bottles planted with bovine tubercle bacilli in 1920, four cultures (44.4 per cent) were viable in 1932 and grew on subculture.

Twelve strains of the original 1919 and 1920 cultures of 17 different strains of human tubercle bacilli subcultured in 1932 grew on the egg medium and were recovered for study and perpetuated, while, of two bovine strains, one grew and was recovered and was perpetuated.

There was noted a certain parallelism between the successful recovery of the bacilli in viable form and the P_H of the medium at the time of the attempt to grow the bacilli twelve years after planting. None of the transplants grew from bottles in which the acidity of the medium was below P_H 6.1 or the alkalinity above P_H 7.6.

A determination of the approximate number of live bacilli present in the culture masses revealed that about 0.01 per cent of the bacilli in the twelve-year-old culture mass of human tubercle bacilli were still viable, while about 1.0 per cent of the bovine bacilli were still able to grow, conforming to earlier observations by us and other investigators who found bovine tubercle bacilli more resistant to detrimental influences in general than human tubercle bacilli.

It was noted that all of the virulent strains of the twelve-year-old cultures of human tubercle bacilli had not suffered a diminution of virulence during twelve years' residence in the incubator at 37° C., while low-virulent strains remained of low virulence during this time. In contrast, prolonged and repeated artificial cultivation, however, caused a slight but definite diminution of virulence (a slow transition) of human tubercle bacilli in stock cultures. It would appear from this that virulence is a property inherent and unchangeable to individual bacilli but prolonged cultivation on artificial media may cause a perceptible, though not striking, change in the progeny of human tubercle bacilli under the ordinary conditions of laboratory perpetuation on culture media.

These observations also suggest a means for maintaining the original culture of a strain of bacilli as control for tests of methods to enhance or diminish virulence as well as to study other factors of the growth of tubercle bacilli.

The morphologic changes noted in the bacilli in the twelve-year-old cultures conformed to those previously described under the changes of the cytomorphosis of tubercle bacilli (1926).

In correlating these culture findings with in vivo conditions of the survival of tubercle bacilli it is evident that the persistence of the bacilli in the body is dependent upon a number of factors, but prominent among these, aside from the pathogenicity of the bacilli, is the factor of the number of tubercle bacilli introduced into the organs and tissues of an animal. This is evident from the fact that while small numbers of virulent tubercle bacilli in fine suspension are removed from the organs and tissues of the rabbit and dog within about one year, large amounts of even avirulent human tubercle bacilli can persist in the tissues of the dog in viable form for over three and a quarter years without the presence of characteristic histologic evidences of tuberculosis in these tissues.

BLOOD, During the First Year of Life, Merritt, K. K., and Davidson, L. T. Am. J. Dis. Child. **46:** 990, 1933.

A group of normal American infants have been studied with reference to the behavior of their erythrocytes, hemoglobin, reticulocytes, and platelets throughout the first year, also the bleeding and coagulation time on the day of birth.

It is apparent that after a period of initial erythrocyte adjustment of two months' duration a mean value of 4.69 million, with a standard deviation of 0.37 million, may be considered as "normal" throughout the first year. After a similar initial period of adjustment, lasting two months, the mean value for hemoglobin for the first year is 12.6 gm. per hundred cubic centimeters, with a standard deviation of 1.36 gm. From these figures, it would appear that normal infants in New York City do not become anemic, as Mackay stated was the case in 51 per cent of artificially fed infants in London. It would seem that her "standard normal hemoglobin curve" is below what may be considered as "normal" in this country. In using Haden's scale of 15.6 gm. per hundred cubic centimeters, which is equivalent to 100 per cent, the color index is found to be 0.86. With Sahli's scale, 17 gm. per hundred cubic centimeters is equivalent to 100 per cent and the color index is therefore 0.90.

The reticulocytes in the normal infant are found to have a mean value of 3.4 per cent at birth. After a rapid drop through the first month, they reach a mean value of 0.16 per cent, with a standard deviation of 0.12 per cent, which is considered normal throughout the first year.

Blood platelets at birth are found to have a mean value of 227 (±3.0) thousand. After a rise at the third month, their mean value throughout the remainder of the first year is 344 thousand, with a standard deviation of 66 thousand. The figures at birth are lower than those of other investigators, and rise less rapidly.

Values for the bleeding time at birth are found to be from one-half to two and one-half minutes. The coagulation time at birth is from two to four minutes.

NOTE: Since this article was accepted for publication, Mackey has published an article, "The Normal Hemoglobin Level During the First Year of Life—Revised Figures," in which it was stated that owing to necessary restandardization of her hemoglobin apparatus, it is necessary to add 7 per cent to each value quoted in her monograph (1931).

LIVER FUNCTION, In Catarrhal Jaundice, Jolliffe, N. Am. J. M. Sc. **186:** 640, 1933.

In 19 cases of the type customarily regarded as catarrhal jaundice it has been found that a definite pathologic and physiologic syndrome was present in 16 of the 19. This consists of a regurgitational jaundice which runs through three distinct stages:

a. An "obstructive phase" with an average duration of eleven days in which there is a progressive deepening of the icterus, absent or mere traces of urobilin in the urine, a retention of bromsulphalein paralleling the degree of jaundice and, usually (87.5 per cent), a positive levulose tolerance test.

b. A "critical phase" with an average duration of four days in which urobilin suddenly appears in the urine in high normal or definitely elevated amounts, a coincident abrupt fall in serum bilirubin and bromsulphalein retention.

c. A "recovery phase" with an average duration of nine days during which time the serum bilirubin falls within the level of latent icterus, urobilin is present in the urine in normal amounts and the tolerance to levulose and the retention of bromsulphalein return to normal.

The term "catarrhal jaundice" should be limited to subjects showing this pathologic and physiologic syndrome.

The 3 remaining cases were of distinctly different pathologic and physiologic types. It is suggested that these types may be appropriately designated as acute hepatitis of unknown etiology with, as the case may be, retentional or regurgitational jaundice.

BLOOD SUGAR, In Schizophrenia, Freeman, W. Am. J. M. Sc. **186**: 621, 1933.

A study is presented of the fasting blood sugar levels as disclosed by 6 samples taken at standard intervals from 59 male schizophrenic patients over a period of six and one-half months.

In 95 per cent of 347 determinations the values lay between the conventional limits of normality, namely, 80 and 120 mg. per 100 c.c. The average value was 96.6 as compared with an average of 95.4 in 31 normal control subjects studied by the same technic. Eight readings were obtained in the hypoglycemic and 8 in the hyperglycemic ranges.

The variation among the schizophrenics was higher than that in the controls, as was shown by the coefficients of variation, 11 and 6.9 per cent, respectively.

The samples were collected in pairs at fifteen-day intervals with intervening periods of two and one-half months. There was a consistent drop in the average blood sugar level from period to period, the first being 99.3 and the third 93.1 mg. per 100 c.c. These findings suggest that habituation with a presumable lessening of emotional reaction to the test played a part in determining the sugar level.

The range of difference in consecutive tests also decreased consistently from one period to another; in the first the average difference was 10.6 and in the last 8 mg. per 100 c.c.

No significant correlation could be recognized between the blood sugar level and the age, period of hospitalization, or severity of the psychosis.

Of the subgroups, the hebephrenics showed the highest average level, namely, 99.7 mg., and the catatonics the lowest, namely 92.7 mg.

DYSENTERY, Amebic, Unusual Experience With. Ikeda, K. J. A. M. A. **101**: 1944, 1933.

Nine cases of amebic dysentery were recognized within a period of forty-seven days in a general hospital of an average size, in a northern city of 250,000 population, in the United States. This is a unique experience. Infection in all nine cases was definitely traced to Chicago, which demonstrates, probably, a widespread dissemination of this disease throughout the country as a direct result of the Chicago epidemic.

The presenting symptoms show a wide variation, depending on the character of the attack and on the stage of the disease.

The initial symptoms, when elicited, are not always identical. There are atypical cases in which the first symptoms may be misleading or so insignificant and commonplace that no medical relief is considered necessary by the patient.

There is danger of a false positive report in the laboratory diagnosis of this disease by the inexperienced. Careful and painstaking search for the organism should be undertaken in suspected and neglected cases.

Roentgen examination may be of value as an aid in differential diagnosis.

Amebas were demonstrated in a section of a piece of tissue from the rectum, in a case suggesting a malignant growth. The histologic appearance of the lesion presents the characteristic initial changes due to the invasion of *Endamoeba histolytica*.

TUBERCULOSIS, A Hematological Retrospect, Oatway, W. H., Jr. Am. Rev. Tubere. 29: 73, 1934.

The author recapitulates the types of blood picture seen in tuberculosis described by him in 1929 as follows:

1. *Normal.*—The total count and each of the cell-types are within certain normal limits. (Total cells, 6,000 to 9,000; neutrophiles, less than 65 per cent, or 5,000 cells; monocytes, less than 10 per cent, or 700 cells; lymphocytes, more than 25 per cent, or 2,000 cells.) It denotes a lack of pathological activity in the lesion, a controlled condition.

2. *Hyperplastic.*—An increase in the mononuclear leucocytes above normal number (more than 9 per cent, or 700 cells). The total count, lymphocytes, and neutrophiles are of normal numbers. It denotes new tubercle-formation. A fairly passive type of reaction, both clinically and pathologically.

3. *Septic.*—Primarily an increase of the neutrophiles (more than 65 per cent, or 5,000 cells). There is often an increase of the total count (more than 9,000 cells), and monocytes (more than 9 per cent, or 700 cells), with a lymphopenia (less than 25 per cent, or 2,000 cells). It denotes tuberculous abscess-formation in some degree and is the most serious type of count, associated with all of the more active features of the disease. The level of septicity is, in general, an indicator of the magnitude of the disease process.

A lymphocytosis (more than 32 per cent) added to a normal or hyperplastic count was seen in more favorable conditions (resistant). Single counts were of most value in showing the tuberculous condition only at the time of the count. Repeated counts were recommended to show the trend of the disease. Very few counts were "out of focus" with the actual condition of the patients. A deviation occasionally found was a slightly septic picture in a patient apparently handling the lesion well. Two patients had large lesions and only a hyperplastic blood picture. Such cases were left in the balance for a later judgment.

Previous conclusions as to the meaning and the relative seriousness of the types of leucocyte pictures have been somewhat further reaffirmed by this study of the fate of 250 cases after over three years.

The septic count is the most dangerous type of the blood picture, and is present most often when death occurs, when the illness becomes chronic, or when there is need of surgery. The hyperplastic count is less favorable than the normal and less serious than the septic count. Monocytosis adds to the danger of the septic count when present. The normal count is very good to attain, and a lymphocytosis makes it of even better import.

Compression therapy, especially pneumothorax, has often interrupted the prognosis of an unfavorable count, and in spite of its risks and failures has no doubt modified from poor to good the conditions and counts of many patients. Its use most frequently in cases with septic counts has often modified the course of the disease and the prognosis of the blood picture in this group.

There is nothing in this further survey to suggest that the previous limits or interpretation should be changed or ignored. Although the interpretation of the blood count in a tuberculous individual should be limited to his immediate condition, the fate of 250 patients has been consistent, in general, with the portent of three years or more ago. In spite of this prescient value of one blood count, following the condition of the disease by sedulously repeated counts, care in the interpretation, and a constant adherence to the meaning is the recommended use of the procedure, and it will provide a valuable clinical adjunct in diagnosis of the patient's condition and in surgery.

ALLERGY: Recent Immunologic Studies in Hypersensitivity to Tobacco, Sulzberger, M. B. J. A. M. A. **102**: 11, 1934.

The studies reported were carried out, in the main, by means of intradermal tests of the skin with tobacco extracts and with nicotine in cases of thromboangiitis obliterans.

Six concepts of fundamental immunologic nature form the basis of the hypothesis that some cases of certain diseases of the vascular system (thromboangiitis obliterans, angina pectoris and coronary disease), clinically long considered to be in some way connected with smoking, are manifestations of hypersensitivity in certain segments of blood vessels to circulating allergens derived from or contained in tobacco.

Concept 1: There is localized and circumscribed fixed specific hypersensitivity; i.e., a hypersensitivity confined to a certain organ or part of an organ may develop.

Concept 2: There is a predilection of certain allergens to sensitize and elicit reactions in certain tissues; i.e., whereas any allergen may sensitize any part, almost all allergens have their favorite point of attack.

Concept 3: Not only sudden and evanescent reactions are elicited when the excitant meets the specifically sensitized shock tissue, but chronic reactions and permanent organic damage are often caused by one or successive shocks resulting from such encounters.

Concept 4: There is an idiosyncrasy, meaning that certain persons react in a manner different from that of the norm; allergens causing reactions in the idiosyncratic person cause

no reactions in normal persons. Allergens are thus usually of harmless nature and are not poisons or toxins in the pharmacologic or toxicologic sense.

Concept 5: The vascular system in man seems to be peculiarly susceptible to sensitizations and is thus most frequently the seat of reactions. Examples of this are found in many diseases of the skin. The wheal reaction is, in itself, a demonstration of vascular hypersensitivity.

Concept 6: The skin is useful as a test tissue in a great variety of hypersensitivities of internal organs.

The experiments in patients with thromboangiitis obliterans and in controls have brought to light the following data: 78 per cent of the patients with thromboangiitis obliterans tested had positive reactions of the skin to tobacco; 36 per cent of the smokers without thromboangiitis obliterans; and 16 per cent of the nonsmokers gave positive reactions.

The persons afflicted with thromboangiitis obliterans were not atopic, and there were no regularly demonstrable reagins in their serums.

As the positive skin tests in patients with thromboangiitis obliterans correspond with the clinical evidence incriminating tobacco, these results are regarded as highly suggestive that sensitization of the vascular system to tobacco may play a causal or contributory rôle in many cases of thromboangiitis obliterans.

Although tobacco was the principal allergen causing positive reactions of the skin in thromboangiitis obliterans, other allergens and, notably, inhalants also elicited positive responses.

It is assumed that cases of thromboangiitis obliterans must exist in which other excitants and not tobacco are the major factors.

That part of the tobacco which elicits the skin reaction of hypersensitivity is coctostabile and thermostabile and is not destroyed by ultraviolet rays or x-rays in the dosages employed. It is not nicotine.

Nicotine is of little or no importance in sensitizations to tobacco. If the diseases under discussion are sensitizations to tobacco, nicotine is, in all probability, of little or no importance in their causation.

It is hoped that the new immunologic approach and the employment of animal sensitizations will lead to the experimental reproduction of certain diseases clinically attributable to damage by tobacco. As is well known all toxicologic attempts to do this have failed.

One of the objects of such experimental studies would be to isolate and, eventually, to eliminate the actual sensitizing factor or factors from tobacco.

LIVER FUNCTION, In Advanced Pulmonary Tuberculosis, Steidl, J., and Heise, F. H. Am. J. M. Sc. **186:** 631, 1933.

In a series of 23 patients having advanced pulmonary tuberculosis all but one gave pathologic readings in one or more of a group of 5 tests for hepatic function. Of the tests used, the cinchophen test, the icterus index and the galactose test were the most sensitive in the order named.

The Journal of Laboratory and Clinical Medicine

VOL. 19 ST. LOUIS, MO., APRIL, 1934 NO. 7

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Entered at the Post Office at St. Louis, Mo., as Second Class Matter

EDITORIAL

The Epidemiology of Amebic Infection

WE HAVE good reason to hope that the great interest in amebiasis, stimulated by the recent Chicago epidemic will be productive of a greatly increased knowledge concerning the epidemiology of this condition. The general understanding based upon reports in the current medical literature is that the Chicago epidemic was the result of food infection following contamination of the food by food handlers, either healthy carriers or individuals mildly ill with amebic colitis. The official report later incriminated defective plumbing systems in the old hotels, in which there was some sort of cross connection between the sewage system and the water supply, such that, with overload, the water supply could become contaminated.

There can be no doubt in view of the reports by Bundensen, of the extremely high incidence of carriers of *Endameba histolytica*, in the hotels chiefly responsible for the epidemic that food contamination played a part, possibly also infected water supply.

The review of the measures for the control of amebic dysentery recently enumerated by McCoy are of great interest in connection with the epidemic. After emphasizing that we do not have sufficient information concerning the factors governing transmission, on which to base authoritative measures, and that the present facts do not justify some of the various measures being put into effect, McCoy summarized present epidemiologic information as follows:

There is very little evidence that the cases originating in Chicago have led to any considerable spread of the infection in other communities into which the infective individuals have gone.

Carriers do not appear as much of a menace as they had been thought to be. There is no clear evidence that carriers, even among food handlers, are important in transmission.

Large scale control of spread by detection of carriers and their removal from food-handling groups appears not practicable.

There is no need for isolation of clinical cases beyond that necessary for the benefit of the patient. There is no need for isolation of carriers.

In the absence of modern methods of sanitary sewage disposal precautions should be taken to prevent water supply contamination and fly contamination. Otherwise no precautions need be taken.

Persons exposed to clinical cases or carriers need no particular attention.

Measures which should be taken by health officers consist in calling the attention of practicing physicians to the importance of recognizing dysentery; insistence upon reporting all cases of dysentery; distinguishing between the amebic and bacillary forms; the provision of facilities for diagnosis; educational measures among food handlers with regard to cleanliness; feces examination of food handlers, measures to prevent possible contamination of drinking water supply, especially in hotels and public eating places.

Spector and Buky report a laboratory investigation in which feces containing ameba histolytica was allowed to dry upon the hand, to determine the viability of cysts. They conclude that the majority of cysts under the circumstances of the experiment were dead at the end of five minutes and that it was very exceptional for any to survive beyond ten minutes.

We infer that the public health service in the discussion just reviewed, are not taking the attitude of the town crier, "twelve o'clock and all's well." Instead, McCoy has presented an analysis of the actual facts as we know them today, with what conclusions are justified by those facts. The reader who does not realize this might be lulled into a false sense of security. The Chicago epidemic was very real, has already caused a very respectable number of deaths, and, as the years go on, will probably be responsible for a great deal of individual illness, the cause of which may remain undetermined. The disease is of sufficient importance, that all promising measures should be taken to prevent its spread, even though some of them may not be completely justified by the facts. Of course one recalls that entire Cuban villages were put to the flame, to destroy yellow fever, which nevertheless returned the next year on schedule. But such useless destruction is not a phase of the prevention of amebic infection.

The clinician is possibly in a better position to study the family incidence of amebiasis than the public health man, and it is this phase of the epidemiology that we would prefer to stress. The reports in the literature together with the experience of all clinicians who see any amount of amebic infection, offer convincing evidence of contact transmission in the family. It seems to us that for the present at least every possible precaution should be taken to recognize amebic infection among food handlers, both in public eating places and in the home, and to prevent this source of food contamination.

Craig, Kofoid and others have for years been preaching the wide distribution of this malady. To an extent they have been crying in the wilderness, but the Chicago epidemic has fully justified their contentions.

Craig emphasizes that, although infection with ameba histolytica is more prevalent in the South than in the North, scarcely a state in the country is free from it, and a conservative estimate indicates that between 5 and 10 per cent of the population of the country, as a whole, harbor the parasite. The majority of infected individuals in this country do not develop frank amebic dysentery, as they do in the tropics.

He states that the cysts are the infecting agents. Under normal conditions the motile trophozoites are destroyed by the gastric juice. Persons suffering with acute amebic dysentery do not have cysts in the stools. They are, therefore, of little importance in contagion. It is the individual with few symptoms and the healthy carrier, who has cysts, who are according to Craig the more important epidemiologic consideration. Acute cases untreated or improperly treated, become carriers of cysts.

He finds that transmission through contaminated water supply is common in localities without modern supply and plumbing systems, particularly when wells, springs, and tanks are depended upon for water. The cysts may live for days and even weeks in water, depending upon the temperature and the number of bacteria present. This method of transmission is of importance in this country only in rural districts. "Wherever sanitation and personal hygiene is poor, amebiasis is very prevalent, whether in the tropics, subtropics, or the temperate zones."

Human excreta used for fertilizer is more of a factor in the Orient than in this country. The cysts will remain viable in moist feces for as long as two weeks. Craig also believes that fly contamination is a distinct factor.

"The transmission of the infection by food handlers is practically a certainty unless the greatest care is taken regarding personal hygiene and the cleanliness of the hands of all who handle food. The incidence of infection in food handlers employed in public eating places is often very high, due to their close association and the constant intake of the cysts in food which they themselves contaminate, and one infected food handler may infect many others with whom he is associated, until the vast majority of the food handlers in a particular institution may show infection."

Craig also emphasizes that there has been no convincing evidence of variation in virulence with different strains of *Endameba histolytica*. Walker and

Sellards successfully produced acute amebic dysentery in human volunteers, with cysts obtained from symptomless carriers.

During the next few years it is obligatory upon all clinicians and clinical laboratory workers to emphasize and reemphasize the importance of careful study of every patient with indefinite or unidentified gastrointestinal symptoms, particularly colonic symptoms, with the possibility of chronic low grade amebic infection in mind. Fortunately, two reliable methods are available, direct examination for motile ameba and for cysts, and the complement fixation reaction which has been developed by Craig.

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—*W. T. V.*